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Vaccinia expression of *Mycobacterium tuberculosis* Antigen 85 and ESAT-6 secreted proteins

Thesis submitted to the University of London in fulfilment of the requirements for the Doctorate of Philosophy

1998

by Adam S Malin

**Department of Infectious and Tropical Diseases
London School of Hygiene & Tropical Medicine
Keppel Street, London, WC1E 7HT**



Numerous
Originals in
Colour



Very
Tightly
Bound

Abstract

Substantial evidence has implicated a role for CD8⁺ T cells in protective immunity against tuberculosis. Recombinant vaccinia (rVV) expressing *Mycobacterium tuberculosis* (MTB) proteins could be used, both as tools to dissect CD8⁺ T cell responses and, in attenuated form, as candidate vaccines. A panel of rVV expressing immunoprotective secreted proteins of MTB have been constructed. An initial rVV employed the vaccinia early/late p7.5 promoter and a gene encoding the 6 kDa early secretory antigenic target 6 protein (ESAT-6). Fidelity of cloning was verified by sequencing, Southern blotting and reverse-transcription polymerase chain reaction. However, attempts to identify protein expression using immunoblotting were unsuccessful.

In order to overcome problems of low level expression, three further strategies were employed. These included: (i) expressing three different MTB genes encoding the Antigen 85 (Ag85) complex (30-32 kDa), as well as ESAT-6, (ii) comparing p7.5 with the more powerful T7 promoter, and (iii) use of a eukaryotic signal sequence belonging to human tissue plasminogen activator (tPA). tPA fusions were created by modifying the insertion plasmids, p1108 (p7.5) and pWR510 (T7), prior to gene cloning. Nineteen rVV were constructed. Protein expression was identified for all rVV except Ag85C constructs. Surprisingly, the T7 hybrid system did not improve protein expression. However, addition of the tPA signal sequence produced a marked enhancement of expression. Three higher molecular weight 85B glycoforms were expressed by rVV-tPA-85B, but not rVV-85B. *In vitro* coupled transcription-translation, in the presence of microsomes, gave further support for tPA-mediated membrane translocation and glycosylation.

rVV-tPA-85A was used to infect human autologous macrophage as target cells in a 6 hour cytotoxic T cell (CTL) assay. Preliminary results suggested specific lysis of target cells using live BCG-stimulated T cells. Thus, these rVV represent powerful tools to investigate the role of CD8⁺ T cells in immunity to tuberculosis. In addition, they offer a potential strategy towards designing a vaccine against tuberculosis.

"Now Kālāmas, do not ye go by hearsay, nor by what is handed down by others, nor by what people say, not by what is stated on the authority of your traditional teachings."

"But Kālāmas, when you know of (it) yourselves..."

Buddha's discourse to the Kālāmas of Kesaputta. Anguttara-Nikāya. I. 188. Pāli Canon. F L Woodward's translation, p 283. London: Geoffrey Cumberledge. 1948.

IN MEMORY OF MY MOTHER
WHO DIED ON OCTOBER 31 ST, 1995.

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Glossary of abbreviations

1°	primary
2°	secondary
Ag85	one or more of three genes encoding the Ag85 proteins (85a, 85b & 85c)
Ag85	Antigen 85 protein(s) - a complex of 3 mycobacterial secreted proteins (85A, 85B & 85C)
APC	antigen presenting cell
ATP	adenosine triphosphate
β-gal	beta-galactosidase
β ₂ m	beta-2-microglobulin
BCG	<i>Mycobacterium bovis</i> bacille Calmette-Guerin
BCL	EBV-transformed B cell lines
bp	base pair
BSA	bovine serum albumin
BUdR	5-Bromo-deoxyuridine
CF	culture filtrate
CFU	colony forming units
CMI	cell-mediated immunity
CPE	cytopathic effect
CTL	cytotoxic T lymphocyte(s)
DAPI	4,6-diamidino-2-phenylindole (stains cell nuclei and produces blue fluorescence)
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulphoxide
DN	double negative
DNase	Deoxyribonuclease I
dNTPs	deoxynucleotide triphosphates
DTH	delayed-type hypersensitivity
EBV	Epstein-Barr virus
ECL	Enhanced Chemiluminescence - trademark for a signal detection system used in immunoblotting protocol (Life Sciences)
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol bis (2-amino ethyl ether) - N, N, N', N'- tetraacetic acid

EMCV	encephalomyocarditis virus
ESAT-6	early secretory antigenic target protein
<i>esat-6</i>	gene encoding ESAT-6
FCS	fetal calf serum
FITC	fluorescein isothiocyanate (produces green fluorescence)
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	good manufacturing practice
<i>gpt</i>	guanine phosphoribosyl transferase gene
HBSS	Hanks Balanced Salt Solution
HRP	horseradish peroxidase
hsp60	60 kDa heat shock protein
IFN- γ	interferon-gamma
IFN- γ R	interferon-gamma receptor
IL-2	interleukin-2
iNOS	inducible nitric oxide synthase
kb	kilobase pair
kDa	kilodalton
KO	knockout
λ (in tables)	lamda <i>EcoRI/HindIII</i> digest molecular weight marker
L-glu	L-glutamine
<i>LacZ</i>	gene encoding beta-galactosidase
LAM	lipoarabinomannan
LB	Luria broth
LMP	low melting point
mAb	monoclonal antibody
MHC	major histocompatibility complex
MM	<i>Mycobacterium marinum</i>
moi	multiplicity of infection
MPA	mycophenolic acid
MTB	<i>Mycobacterium tuberculosis</i>
MVA	modified vaccinia virus Ankara
MW	molecular weight (or molecular weight marker, in tables)
NEM	N-ethylmaleimide
NO	nitric oxide
NP-40	nonidet P-40
OADC	oleic acid-albumin-dextrose-catalase supplement

p/s	penicillin/streptomycin
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units
pi	post-infection
PMSF	phenyl methyl sulphonyl fluoride
PPD	tuberculin purified protein derivative
rFPV	recombinant fowlpox virus
RIPA	radioimmunoprecipitation buffer
rMVA	recombinant modified vaccinia virus Ankara
ROI	reactive oxygen intermediates
RT	reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction
rVV	vaccinia recombinant(s)
s/n	supernatant
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SSI	Statens Seruminstitut, Copenhagen
ST-CF	short term culture filtrate
T25, T75 & T175	three different cell culture flask sizes with culture surface areas of 25, 75 and 175 cm ² respectively
TAE	Tris/acetic acid/EDTA buffer
TB	tuberculosis
TK ⁻ 143	a human osteosarcoma cell line with functional deletion of the thymidine kinase locus
TNF- α	tumour necrosis factor-alpha
tPA	tissue plasminogen activator (text)
-tPA	tissue plasminogen activator signal sequence (part of rVV or plasmid)
TPCK	N-tosylamide-L-phenylalanine chloromethyl ketone
UV	ultraviolet
vTF7-3	rVV expressing T7 polymerase
VV	vaccinia
wt	wild type
ZN	Ziehl-Neelsen

1 Introduction

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1.1 AIMS AT OUTSET OF PROJECT

1.1.1 Broad, long term objectives

At the outset of this study, it was proposed to utilise vaccinia recombinant DNA technology as a tool to assess major histocompatibility complex (MHC) class I antigen presentation of *Mycobacterium tuberculosis* (MTB) secreted proteins to human T cells. Recombinant protein expressed in this way would permit presentation of MTB antigens in a form suitable for recognition by CD8+ T cells. Furthermore, given that recombinant vaccinia would present antigen in the context of MHC class II as well as class I, this could give rise to a balanced CD4+ and CD8+ T cell immune response. Thus, it was also proposed to assess this antigen delivery system as a potential vaccine against tuberculosis (TB).

1.1.2 Specific aims

From the known available gene sequences of MTB, four genes were selected. These encoded the highly immunodominant secreted proteins: Antigen 85A, 85B and 85C (30-33 kDa) and the early secretory antigenic target protein (ESAT-6) (6 kDa). These genes were to be obtained, as well as reagents for detection, from collaborators, Drs K Huygen and J Content for the genes encoding Ag85 and Dr P Andersen for the gene encoding ESAT-6.

Vaccinia recombinants (rVV) expressing these antigens were to be constructed for human cytotoxic T cell assays to be performed as part of this study. In addition rVV were to be supplied to collaborators at the Pasteur Institute, Brussels (rVV-Ag85 constructs) and the Statens Seruminstitut, Copenhagen (rVV-ESAT-6) for murine immunology.

1.2 BACKGROUND AND SIGNIFICANCE

1.2.1 A need for a new vaccine

Despite widespread use of *Mycobacterium bovis* bacille Calmette-Guerin (BCG) vaccination, MTB remains the single biggest infectious killer worldwide (Bloom and Murray, 1992). Globally, it is also the major pathogen associated with HIV disease (Bloom and Murray, 1992; De Cock et al 1992). The available vaccine, BCG, has been given to more people than any other vaccine (Bloom and Fine, 1994). Yet the vaccine offers protection only against disseminated childhood TB, and not the more prevalent (and infectious) adult pulmonary disease (Fine and Rodrigues, 1990). Indeed, in large studies in both southern India ("The Tuberculosis Prevention Trial", 1980) and Malawi (Ponnighaus

et al. 1992), BCG was ineffective as a vaccine against pulmonary TB. Alternative vaccine strategies are therefore urgently required

1.2.2 The failure and success of BCG

The reasons for the failure of BCG remain unclear. Immunity to TB is poorly understood both at a cellular and molecular level (Bloom and Murray, 1992; Orme et al. 1993). It is possible that BCG's ability to protect against initial infection may wane with time. Alternatively, BCG may be unable to prevent the establishment of dormant infection, so giving the potential for reactivation later in adult life (Malin and Young, 1996). One mechanism could be that BCG prevents blood-borne spread (the 'bacillaemia'), but not seeding in the lungs (Wiegshauss and Smith, 1989). This could also explain its protective efficacy against childhood miliary disease and TB meningitis

These explanations, however, do not account for the enormous variation seen in different settings. For example, in 11 controlled trials of BCG performed in four continents, vaccine efficacy varied between 0 and 80%. In North America and the UK, in a younger age population, there was a protective efficacy ranging from 60 to 80%. These studies included: British school children (aged 14 years), North American Indians (two studies: <3 months and 0-19 years), a study of infants in Chicago (< 3 months) and young adults in Haiti (<20 years). This is in stark contrast to zero efficacy seen in two controlled trials in Chingleput, India (> 1 year) and Georgia, United States (6-17 years) (Rodrigues and Smith, 1990; Bloom and Fine, 1994), where the vaccine population tended to be older. Whilst age of vaccination could explain some of the inter-study variation, this was not supported by observational studies (case-control and contact studies) which showed a similarly wide variation in protective efficacy, not accountable by age alone (Fine and Rodrigues, 1990). This overall pattern of varying efficacy has led one researcher to comment that protection appears to be less effective the closer one gets to the equator (Prof P. Fine, personal communication). Several additional factors could account for the large variation between the studies: (i) methodological flaws including poor randomisation of vaccine and placebo groups (susceptibility bias), unequal-equal follow up (surveillance and diagnostic-testing bias) and non-blinded evaluation of subjects i.e. knowledge of their vaccination status (diagnostic interpretation bias); (ii) heterogeneity between different vaccine strains; (iii) differences in virulence of MTB strains; (iv) variation in exposure to MTB i.e. cases may be predominantly exogenous (where level of exposure is high) or predominantly endogenous

(where level of exposure is low); (v) interaction with environmental mycobacteria; and (vi) genetic differences between subject populations. No single factor can explain both the failure and success of BCG. However, there is increasing evidence to support a contributory role for both genetic and environmental influences. Elucidation of these factors will support rational design of a future vaccine.

1.2.2.1 Genetic influences on immunity

There is no direct evidence to suggest a genetic cause which would explain variation in vaccine efficacy. However, compelling evidence exists for genetic influences affecting both susceptibility to TB and disease severity. By analogy, it would seem reasonable that these or other genetic factors could affect vaccine efficacy. Evidence in favour of an overall genetic influence is uncontested (Newport and Blackwell, 1997). The key points are summarised below.

- 1) In 1926, 251 infants from Lubeck in Germany were inadvertently immunised with a virulent strain of MTB instead of BCG. The responses to infection were markedly different; 47 developed no detectable disease, 127 developed radiologically-confirmed disease and 77 died ("Anonymous", 1935).
- 2) Several geographically remote populations not previously exposed to MTB were highly susceptible to TB. The course of disease was often rapidly progressive and resulted in exceptionally high mortality rates (Stead, 1992). For example, at the turn of the century, the Canadian Indians of the Qu'Appelle valley were first exposed to MTB resulting in a very high initial annual mortality of 10%. Following several generations of exposure, the annual death rate fell to 0.2%. This finding supported the concept of genetic selection in favour of those less susceptible to severe disease (Chan and Kaufmann, 1994). A confounding factor included the possibility of MTB strain differences, the Indian population being exposed to less virulent strains over time.
- 3) Twin studies have provided clear evidence for a genetic influence showing that a monozygotic twin of a TB case is much more likely to develop disease than a dizygotic one (Comstock, 1978).
- 4) There is also evidence to support a racial difference between black and white populations. For example, one study examined infection rates, as judged by skin test conversion, in nursing homes across Arkansas in the US. In this study, blacks were twice as susceptible to infection as whites. However, of those infected, there were

no racial differences in terms of progression to active disease (Stead et al. 1990). Whilst such a study provides suggestive data, confounding factors such as nutritional status and underlying disease, such as diabetes, were not addressed.

Despite many studies, there is no clear evidence in favour of a strong HLA link with susceptibility to TB (Mehra, 1990; Pitchappan, 1990). However, the data is better for non-HLA gene associations and disease susceptibility. Evidence from a mouse model (Blackwell, 1996) and some Maltese families (Levin et al. 1995; Newport et al. 1996) have shown that a single gene can have a strong influence on disease susceptibility. It has been difficult to show the same effect produced by a single gene at a population level. The strongest evidence comes from a population-based case-control analysis of TB in The Gambia. A significant number of TB cases were homozygous for a variant allele in the 3'-untranslated region of the vitamin D receptor gene. The same finding was not identified in a parallel study in India (Bellamy et al. manuscript submitted).

1.2.2.2 *Environmental influences on immunity*

Environmental factors, such as the effects of host nutritional status (Chan et al. 1996; Chandra, 1996) or prior infection with environmental mycobacteria (Stanford et al. 1981; Stanford et al. 1987), could explain varying patterns of vaccine efficacy in different regions. In a recent study assessing disease susceptibility, mice fed on a 2% protein diet, as compared with littermates receiving a 20% protein diet, rapidly succumbed to infection with MTB (Chan et al. 1996). One could extrapolate that chronic malnutrition would also lead to a reduction in vaccine efficacy (Chandra, 1996).

Prior infection with environmental mycobacteria could also generate an altered immune response affecting subsequent BCG immunisation (Stanford et al. 1981). This impact could be beneficial, neutral or deleterious. For example, the environmental mycobacteria might: (i) induce an initial immune response followed by boosting with BCG, thus conferring greater protection than produced by BCG alone; (ii) confer similar protection to BCG, with nothing further added by BCG vaccination (Wiegshaas and Smith, 1989); or (iii) confer no protection and inhibit the protective efficacy of BCG (Stanford et al. 1981). Furthermore, it is possible that immunological interference may be dependent on the mycobacterial species (Stanford et al. 1981; Stanford et al. 1987; Wiegshaas and Smith, 1989).

Wiegand and Smith (1989) have argued that the failure of BCG to protect against TB in the Chingleput trial is due to the second of these scenarios - environmental mycobacteria confer some protection and nothing further is contributed by BCG. They have commented that the number of cases in the first 7½ years of the trial was smaller than expected ("The Tuberculosis Prevention Trial", 1980). Moreover, 95% of the population over the age of 10 showed previous exposure to environmental mycobacteria. This suggested to the authors that the heavy environmental mycobacterial exposure would have induced protection against TB, such that subsequent BCG vaccination could confer little in addition.

Whilst environmental mycobacteria may have contributed to the limited success of previous vaccination programmes, the advent of neonatal vaccination as part of the Expanded Programme of Immunisation would greatly limit this influence. However, other environmental issues, such as those related to poverty and underlying disease, will still continue to influence variability in vaccination efficacy throughout the world.

1.2.3 An ideal TB vaccine

An ideal TB vaccine would be given at birth, confer life-long protection against disease and be delivered as a non-living subunit formulation (with a view to safety and quality control). Possible alternative profiles for new vaccines include a "booster" vaccine that could be given to young adults who are a high-risk age group, a "transmission-blocking" vaccine which would decrease sputum smear-positivity, or an "immunomodulating" or therapeutic vaccine (Stanford and Grange, 1993).

A sub-unit vaccine approach is attractive in terms of both quality control and safety. However, in order to pursue a rational design strategy, it is necessary to know both the immune components necessary for protective immunity and the antigens required to induce this state (Malin and Young, 1996).

1.2.4 MTB and the macrophage - a unique intracellular niche

MTB is a facultative intracellular parasite with the capacity to survive both intra- and extracellularly. It usually resides within mononuclear phagocytes (monocytes and macrophages) where replication may occur. The microorganism utilises multiple receptor-ligand interactions to mediate attachment and phagocytic uptake. For example, the MTB cell wall-associated lipoarabinomannan (LAM), the mycobacterial equivalent to

lipopolysaccharide, can interact with the mannose receptor of the host cell. Additional cell receptors include: collectins such as protein surfactant A (Downing et al. 1995) and mannose-binding lectin and also the complement receptors, CR1, 3 and 4 (Chan and Kaufmann, 1994). Once in the mononuclear phagocyte, the bacilli may multiply slowly, with a generation time of only 12-24 hours or alternatively, remain quiescent for years. Thus, the disease is characteristically chronic, with an insidious onset and slow progression. Furthermore, an infected individual may remain latently infected and free from symptoms for decades until subsequent disease reactivation. MTB possesses a number of survival strategies to avoid destruction within what would normally be a hostile intracellular environment. It resides within its own phagosomal compartment (the 'TB phagosome'), avoids phagolysosomal fusion (Clemens and Horwitz, 1995), and prevents phagosomal acidification by exclusion of the vesicular proton pump ATPase (Sturgill-Koszycki et al. 1994). This latter property is thought to be a key mechanism through which MTB avoids intracellular destruction.

Macrophage activation and destruction of intracellular pathogens include a variety of mechanisms, in particular the generation of reactive oxygen intermediates (ROI) and nitric oxide (NO). In the case of ROI, MTB possesses several products which either inhibit the respiratory burst that generates ROI or neutralise their effects. These include sulphatides, LAM and the secreted protein, superoxide dismutase (Clemens, 1996). The protective role for NO production in murine antimycobacterial immunity has been established, however, its role in human immunity remains moot. In murine models, IFN- γ -mediated activation of macrophages led to up-regulation of inducible nitric oxide synthase (iNOS) resulting in control of infection. The addition of iNOS inhibitors resulted in exacerbation (Kamijo et al. 1994). Furthermore, disruption of the IFN- γ gene (Flynn et al. 1993) or its receptor (Kamijo et al. 1993) also resulted in more severe infection and a reduced ability to produce NO during infection. However, iNOS has not been consistently demonstrated in human monocyte-derived activated macrophages, although it has been identified in alveolar macrophages from patients with TB (Nicholson et al. 1996). Further data are required to elucidate its importance.

Virulent mycobacterial species, such as MTB and *M. leprae*, possess an additional survival feature. The terminal arabinose side-chains of MTB LAM are capped with multiple units of mannose (Man-LAM). Fast-growing, avirulent mycobacterial strains lack this mannose

capping and their LAM, unlike Man-LAM, can act as a powerful inducer of TNF- α and many of the mRNA molecules associated with macrophage activation such as NF- κ B, KC and C-fos transcripts. Thus, the virulent mycobacteria could be perceived as having developed an inert form of LAM, permitting prolonged host cell survival (Kumararatne and Dockrell, 1997).

1.3 IMMUNITY TO TB

Over a third of the world's population are infected with MTB, yet of those infected, more than 90% will never develop overt disease (Bloom and Murray, 1992). If an individual with latent TB infection becomes immunosuppressed, for example with HIV infection, their likelihood of developing clinical TB increases from about 5% per life-time to 8% per year (Raviglione et al. 1992). This suggests that infection with the virulent form of the organism successfully induces a 'suppressive', but not sterilising, immune response in most immunocompetent people. Why some 5-10% of the infected population fail to develop long-lived protective immunity is complex and imperfectly understood. What is known about the immune response is derived from a variety of sources including animal models of infection and protection, human *in vitro* studies and human population-based studies of skin test reactivity, MTB infection and disease.

1.3.1 A pivotal role for CD4+ IFN- γ secreting cells

CD4+ IFN- γ -secreting T cells are pivotal in immunity against mycobacterial infection in murine models (Orme et al. 1993a). Key evidence comes from protection studies. Orme (1987) showed that CD4+ T cells from MTB-infected mice could adoptively protect sublethally irradiated mice from MTB challenge. Pedrazzini and colleagues (1987) offered further support by showing that CD4+, and not CD8+, T cell depletion *in vivo* resulted in loss of control of BCG replication in mice. In addition, mycobacterial specific T cell cloning studies demonstrated that CD4+ Th1-type T cell clones could adoptively transfer DTH activity (Pedrazzini and Louis, 1986; Boom et al. 1987). However, it should be noted that DTH activity is not synonymous with protection (Orme and Collins, 1984; Fine et al. 1994). In humans, evidence for the importance of Th1-like CD4+ T cells is circumstantial. Firstly, there is a strong correlation between CD4+ T cell responsiveness and the extent of disease. For example, localised disease such as TB pleurisy is associated with strong CD4+ IFN- γ responses (Barnes et al. 1990), whilst disseminated disease is not (Kumararatne and Dockrell, 1997). Secondly, HIV infection results in progressive CD4+ lymphopenia; this

infection represents by far the most potent risk factor for the development of TB with a relative risk of 6-100 as compared with HIV-negative individuals (Nunn et al. 1994). Furthermore, a study from Côte D'Ivoire showed a inverse correlation between CD4⁺ count and the extent of MTB dissemination and overall bacillary load (Lucas et al. 1994). A third piece of indirect evidence comes from a study of Maltese children with an autosomal recessive immunological defect which results in fulminating non-tuberculous mycobacterial infection. Using microsatellite and sequence analysis, a mutation in the IFN- γ receptor 1 (IFN- γ R 1) was identified (Newport et al. 1996). Previously, a study using mice with disruption of the IFN- γ R gene also showed a marked increase in susceptibility to disease (Kamijo et al. 1993). In a sense, these affected children represent a human counterpart to the IFN- γ R KO mice.

The murine adoptive transfer experiments suggest that protection could be mediated entirely by an interaction between CD4⁺ Th1-type T cells and activated macrophages. Whilst the human data offers support for the importance of this T cell population, their relative contribution remains unknown. Mice are relatively resistant to MTB and do not develop cavitation or caseous necrosis, the hallmark of human disease (Collins, 1984). Furthermore, an *in vitro* study comparing the effect of IFN- γ when added to murine or human macrophages infected with MTB showed that the activated murine macrophages led to a marked reduction in numbers of the organism (Rook et al. 1986a). In stark contrast, IFN- γ had either no effect or a negative effect on MTB-infected human macrophages. Thus, a CD4⁺ IFN- γ secreting T cell population is unlikely to be the single key immune contributor to protection against human disease.

1.3.2 Are CD8⁺ T cells important?

1.3.2.1 Identification hampered by theory and methodology

There is still no satisfactory *in vitro* model to assess immunological correlates of protection in humans. This failure may be a consequence of the limited number of immune responses measured, the predominance being towards CD4⁺ T cell proliferative and cytokine responses. T cell cytotoxicity has been measured in mouse models of TB and researchers have demonstrated a role for both CD4⁺ T cells (Orme, 1987; Pedrazzini et al. 1987) and CD8⁺ T cells (Orme and Collins, 1984; De Libero et al. 1988). However, few studies have evaluated the human CD8⁺ T cell response to TB (Orme et al. 1993a).

Until the development of knockout mouse models, there was little inclination to investigate CD8⁺ T cell immunity. This may have arisen for two historic reasons. Firstly, MTB is thought to be confined to a unique vacuolar compartment, there being little evidence for escape into the cytoplasm (Sturgill-Koszycki et al. 1994; Clemens and Horwitz, 1995; Moreira et al. 1997). By inhibiting phagolysosomal fusion and the proton ATPase pump (Sturgill-Koszycki et al. 1994), MTB can exist, undisturbed, within its own endocytic niche (Moreira et al. 1997). Because of its membrane-bound location, antigen processing was thought to be limited to the MHC class II pathway. Thus, the majority of the T cell immunity would be mediated by CD4⁺ T cells (Kaufmann, 1993). Secondly, this dogma was supported by early data which tended to identify mostly CD4⁺ T cell reactivity (Emmrich et al. 1986; Kaufmann et al. 1987; Oftung et al. 1987; Andersen et al. 1991; Manca et al. 1991; Orme et al. 1992; Orme et al. 1993). However, soluble antigen was used for *in vitro* stimulation of human or murine T cells. This would have favoured the CD4⁺ T cell phenotype (see Section 1.4.1.3). Further evidence has now accumulated to implicate a significant role for CD8⁺ T cells in protective immunity (Flynn et al. 1992; Moreira et al. 1997). In addition, alternative pathways for MHC class I antigen processing have been elucidated, suggesting a mechanism whereby mycobacterial protein can be recognised by CD8⁺ T cells (Kovacsovic Bankowski et al. 1993; Pfeifer et al. 1993; Kovacsovic Bankowski and Rock, 1995). These data are discussed below.

1.3.2.2 Evidence implicating human CD8⁺ T cells in immunity

TB pleural effusions represent a useful source of human activated lymphocytes at the site of disease. Using this cell population, data in favour of a role for CD8⁺ T cells have shown that: (i) these cells are present at the site of infection (Rees et al. 1988; Manca et al. 1991); (ii) CD8⁺ T cell clones derived from these cells recognise mycobacterial antigens (Rees et al. 1988); and (iii) that, in one study of 4 patients, they constituted the majority of T cells present prior to *ex vivo* stimulation (Manca et al. 1991). Whilst this cell population offers easy access for laboratory manipulation, a caveat requiring consideration is that pleural TB represents only one manifestation of the clinical spectrum of disease (Kumararatne and Dockrell, 1997). Typically patients are relatively well, acid fast bacilli (AFB) are difficult to isolate and CMI is strong (analogous with paucibacillary disease found in leprosy). Therefore, one should be cautious about extrapolating these findings to other forms of TB.

Furthermore, human CD8⁺T cells, following 7 days of re-stimulation with live BCG in the absence of exogenous cytokines, could specifically recognise and lyse PPD-pulsed or BCG-infected autologous macrophage target cells in a standard 6 hour cytotoxic T lymphocyte (CTL) assay (Turner and Dockrell, 1996). The cells in this study were derived from BCG vaccinees, however, unpublished data suggests that BCG-specific CTL can also be found in patients with TB (Dr J. Turner, personal communication). Recent unpublished data has demonstrated that an ESAT-6 specific CD8⁺ T cell line will recognise a rVV-ESAT-6 infected B lymphoblastic cell line in an MHC class I restricted manner (see chapter 6).

1.3.2.3 *A β_2 -microglobulin knockout model*

β_2 -microglobulin (β_2 m) constitutes the smaller of two molecules required for functional expression of MHC class I. Thus, targeted disruption of the β_2 m gene in mice (β_2 m KO mice) results in paucity of CD8⁺ T cells due to absence of positive selection (Raulet, 1994). The most cited evidence for CD8⁺ T cell involvement comes from MTB challenge of β_2 m KO C57BL/6 mice (Flynn et al. 1992). This study demonstrated that MHC class I-restricted T cells were essential for protection against a 10^6 colony forming units (CFU) intravenous challenge of MTB (Erdman strain). Within 10 weeks, all β_2 m KO mice died from overwhelming infection, in contrast to their genetically normal littermates (β_2 m +/+) which survived >20 weeks. Organ histology from both groups of mice demonstrated well demarcated granulomas with normal architecture. However, the macrophages from the β_2 m KO mice were stuffed with bacilli and, by 6 weeks post-infection, these granulomas were necrotic and occupied over 70% of the lung volume. For the β_2 m +/+ mice, only 30% of the lung volume was occupied by granulomas and these showed no evidence of necrosis. In contrast, BCG infection resulted in no difference in survival or organ homogenate CFU counts between β_2 m KO and control groups of mice.

In the same study, the researchers also addressed the issue of a protective contribution from cytokines, particularly as CD8⁺ T cells may secrete a large amount of IFN- γ . Stimulation of splenocytes harvested 2-3 weeks post-infection did not result in a significant difference in either IFN- γ or tumour necrosis factor-alpha (TNF- α) production between groups of mice (Flynn et al 1992). This study offered strong support in favour of a contributory role for MHC class I-restricted T cells. However, there are two important issues concerning this study. Firstly, a very large, 'non-physiological', dose of virulent MTB was given to the mice. In humans, most infective doses would occur via the respiratory route with an inoculum of

about 10^1 – 10^3 bacteria. Thus, this system could be testing an immunological function not encountered in a typical human immune response. Secondly, the same result has not been reproduced, at least in the published literature, for CD8⁺ knockout mice. Thus, the evidence for a CD8⁺ T cell contribution remains indirect.

Ladel and colleagues then examined the impact of infection with two different doses of BCG in β_2m KO mice. Using the same 10^6 CFU dose as the previous study, a similar pattern was observed, with no deaths in the β_2m $-/-$ group. However, by increasing the dose threefold, half of the β_2m $-/-$ mice had died by 6 months as compared with no deaths in the β_2m $+/+$ controls (Ladel et al. 1995). This study provided clear evidence for the inoculating dose determining the relative importance of MHC class I-restricted T cells.

1.3.2.4 *Non-peptide antigens and CD1-restricted T cell recognition*

An alternative explanation for the β_2m KO study results could be that the class I-restricted T cell contribution comes from a population of $\alpha\beta$ CD4⁻ CD8⁻ (double negative, DN) T cells recognising non-peptide mycobacterial antigens presented by the non-polymorphic CD1 molecules. The CD-1 molecules are MHC class I-like molecules and would also be absent in β_2m KO mice. Porcelli and colleagues (1992) were able to demonstrate that non-peptide mycobacterial antigen could be recognised by human DN T cells and that this recognition was restricted to CD1b (one of 5 allelic forms). Subsequently, both mycolic acid and lipoarabinomannan (components of the mycobacterial cell wall) were shown to be recognised by DN T cell lines, also restricted by CD1b presentation (Beckman et al. 1994; Sieling et al. 1995). Another member of this CD1 family, CD1c, was also able to present mycobacterial lipids. T cell clones restricted to this molecule possessed a cytotoxic function and were MHC unrestricted (Beckman et al. 1996). Whilst this non-peptide antigen recognition is intriguing, the biological importance has not yet been demonstrated. The DN T cell population constitutes a relatively small proportion of T cells. Furthermore, there is little evidence for *in vivo* clonal expansion in disease models, a characteristic feature seen with both CD4⁺ and CD8⁺ T cells (Manca et al. 1991). A knockout model would help resolve the issue concerning biological importance. However, such a knockout would require disruption of two genes in the mouse.

1.3.2.5 *What is the effector mechanism for CD8+ T cells?*

The effector function of CD8+ T cells could be mediated by target cell lysis leading to death of the intracellular pathogen. This is known to be an important effector mechanism, for example, in the control of *Listeria* (Kaufmann, 1993). An alternative effector function could be provision of cytokines such as IFN- γ in an analogous fashion to CD4+ T cell function. MTB is thought to down-regulate MHC class II on the surface of infected macrophages (Mazzaccaro et al. 1996). Perhaps CD8+ T cells could provide an additional population of immune effectors able to recognise those infected macrophages lacking surface MHC class II molecules and thus provide an alternative source of IFN- γ . Both cytotoxicity and cytokine production could potentially play a role in mediating CD8+ T cell function. Direct evidence for murine CD8+ T cell cytotoxicity (CTL) comes from immunisation of major histocompatibility complex (MHC) class I congenic mice with BCG (Denis et al. 1997). In this study, CD8+ CTL were demonstrated against BCG culture filtrate (CF) antigens using H-2b and H-2bx d haplotypes but not the H-2d haplotype or the MHC class I mutant mouse strain haplotype, B6C-H-2bm13. This suggested that these BCG-induced CTL recognized CF antigens exclusively in association with D(b) molecule. However, evidence from gene knockout experiments suggests CTL activity may not be required (see below) (Cooper et al. 1997; Laochumroonvorapong et al. 1997).

1.3.2.6 *CTL activity does not kill mycobacteria*

Mycobacteria are thought to be killed within highly activated macrophages by a variety of intracellular mechanisms (Section 1.2.4). The effectors which mediate successful macrophage activation and mycobacterial death are not fully understood and there is a clear variation between species (Rook et al. 1986a). Theoretically, the immune control of MTB infection could be mediated entirely by an interaction between cytokine-producing $\alpha\beta$ CD4+ T cells and activated macrophages, as suggested in some mice models (Orme, 1987; Pedrazzini et al. 1987). However, other immunological immune mediators are readily identifiable and include: cytolytic activity, $\gamma\delta$ T cell and $\alpha\beta$ DN T cell responses, and antibody responses. For example, cytolytic activity has been readily demonstrated by both CD4+ and CD8+ T cells (Orme and Collins, 1984; Orme, 1987; Pedrazzini et al. 1987; De Libero et al. 1988). The precise function of these CTL is unknown. There is no evidence suggesting that they reduce mycobacterial numbers. For example, human CTL lysis of BCG-infected macrophages had no effect on mycobacterial growth, even if the macrophages had been previously co-cultured with IFN- γ (Fazal et al. 1995). Furthermore, perforin- or fas-

receptor gene-disrupted mice, were still able to control a challenge with MTB or BCG (Cooper et al. 1997; Laochumroonvorapong et al. 1997). It is interesting to note that in one of the studies, both groups of knockout (KO) mice showed a significant compensatory increase in cytokine levels following mycobacterial challenge (Laochumroonvorapong et al. 1997). This latter point emphasizes the fact that KO mice may develop immune compensatory mechanisms in response to their congenital immunological defect. In principal this problem could be addressed using the bacteriophage-derived Cre-loxP recombination system where the gene deletion can be induced in the fully developed animal (Gu et al. 1994).

Given that CTL function for both CD4+ and CD8+ T cell populations is readily observed, teleologically, one would imagine that these CTL would serve a useful purpose. A possible role could be the release of mycobacteria from effete macrophages unable to destroy the organism. This would permit phagocytosis by macrophages better able to kill or contain mycobacterial growth. An alternative explanation is that the observed lysis may simply represent redundancy in the immune response. This could be likened to the strong antibody response also seen in many mycobacterial infections including TB, which apparently serves no useful function.

1.3.2.7 Alternative pathways of MHC class I antigen processing - Tap-dependent, Brefeldin A sensitive or peptide regurgitation

Whole MTB organisms may not need to enter the cytoplasm for MHC class I expression of mycobacterial proteins. There is good evidence that particulate antigen may be taken up into a vacuolar compartment by professional antigen presenting cells (APCs) and be processed and presented in the context of MHC class I (Kovacsovics Bankowski et al. 1993; Pfeifer et al. 1993; Harding and Song, 1994). For example, chicken egg ovalbumin linked to beads was presented with MHC class I molecules 10⁴-fold more efficiently than soluble ovalbumin; this mechanism was inhibited by cytochalasin B, an inhibitor of phagocytosis. In addition, CD8+ CTL could be induced by immunising mice with coated beads (Kovacsovics Bankowski et al. 1993). Subsequently, the same group showed that the mechanism was dependent on TAP1-TAP2 peptide transporter and inhibited by Brefeldin A (an inhibitor of endoplasmic reticulum/Golgi transport). They concluded that the pathway entailed egress of protein from its vacuolar compartment into the cytoplasm, followed by proteasome complex degradation and processing in a similar manner to other cytoplasmic proteins

(Kovacsovics Bankowski and Rock, 1995). In contrast, ovalbumin-coated beads, or bacterial recombinants expressing an ovalbumin MHC class I restricted-epitope, showed evidence for a Brefeldin A resistant pathway (Pfeifer et al. 1993, Harding and Song, 1994). These authors suggested that this was mediated by peptide regurgitation outside the APC. For example, degraded protein could diffuse across a 0.45 μm membrane to be presented by fixed macrophages of a different MHC class I haplotype (Harding and Song, 1994). The researchers suggested that an alternative antigen processing pathway exists which requires post-Golgi loading of peptide onto MHC class I molecules. It is possible that more than one alternative MHC class I antigen processing pathway exists.

1.3.2.8 Does MTB favour CD8⁺ T cell induction?

Particulate but not soluble exogenous protein can gain access to an MHC class I antigen processing pathway; this applies to membrane-bound intracellular bacteria such as *Salmonella* spp. and *E. coli* (Harding and Song, 1994). It is possible that the same would be true for MTB and other mycobacteria. This hypothesis was tested by Mazzaccaro and colleagues (1996). Using exogenous soluble ovalbumin as a reporter molecule, MTB and BCG were tested for their ability to permit processing of ovalbumin for presentation to MHC class I and/or class II hybridoma cell lines. Live MTB infection was selective for MHC class I presentation, since MHC class II presentation was inhibited. This effect was true only for viable organisms. In addition the processing pathway was dependent on the TAP transporter. BCG produced a similar pattern of MHC class I presentation, but to a lesser extent. The authors suggested that mycobacterial proteins could gain access to MHC class I processing in a manner analogous to *Listeria monocytogenes*. *L. monocytogenes* produces a pore-forming haemolysin, listeriolysin, which allows protein to escape into the cytoplasm. An MTB haemolysin has been identified and its role is under investigation (Dr N. Stoker, personal communication). Whilst the study by Mazzaccaro and colleagues (1996) demonstrated class I-restricted antigen processing of the reporter molecule, ovalbumin, it did not demonstrate that mycobacterial proteins are also subject to the same antigen processing. This issue could be addressed following the development of MHC class I and class II-restricted T cell clones known to recognise specific epitopes within a mycobacterial protein.

It was interesting to note that BCG was also able to permit some MHC class I ovalbumin processing, but to a lesser extent than MTB. This may be important concerning the

moderate efficacy of BCG vaccination. In part this may be due to a failure to induce a strong enough CD8⁺ T cell response. However, some MHC class I mycobacterial antigen processing may occur as human CD8⁺ T cell induction is seen with live but not killed BCG (Turner and Dockrell, 1996).

1.3.2.9 *Animal protection models and CD8⁺ T cells*

Vaccination with a murine transfected tumour cells expressing *M. leprae* hsp 65 conferred protective immunity to a virulent strain of MTB in mice (Silva and Lowrie, 1994). Further protection studies in mice used DNA vaccination. Using a variety of different genes encoding mycobacterial antigens, a similar degree of protection was conferred using DNA vaccination as compared with BCG immunisation (Huygen et al. 1996; Tascon et al. 1996). It is remarkable that expression of single antigens was able to achieve this degree of protection. Due to the nature of the vaccine delivery systems, the tumour cell vaccine as well as the DNA vaccines induce both CD4⁺ and CD8⁺ T cells. It is possible that antigen delivery inducing this balanced response may be a prerequisite for successful immunity against MTB.

1.3.2.10 *The granuloma and control of infection - apoptosis versus CTL*

No single mechanism or mechanisms have emerged to explain how the host combats MTB infection. It could be an error to attempt understanding at a molecular and cellular level alone. Perhaps the key resides within the granuloma, a pathophysiological 'organ' comprising of mycobacteria sealed-off from the rest of the host by mononuclear phagocytes in various states of activation surrounded by a palisade of activated lymphocytes. The central zone may be necrotic with characteristic caseation, the result of tissue destruction and macrophage cell death. This environment could determine the various outcomes associated with infection: (i) destruction of the mycobacteria and subsequent resolution of the granuloma; (ii) containment of mycobacteria and persistence of the granuloma; or (iii) uncontrolled replication of MTB resulting in liquefaction and systemic mycobacterial spread. Accruing evidence has suggested that the form of macrophage cell death may determine the outcome of mycobacterial survival. Two types of cell death are distinguishable: (i) necrosis with disintegration of the cytoplasm, loss of organelles and the nucleus remaining relatively intact; and (ii) apoptosis with nuclear fragmentation as a result of endonuclease activation. Evidence for both types of cell death exists in TB (Kumararatne and Dockrell, 1997). There has been little evidence to demonstrate that cytolysis reduces mycobacterial numbers

(discussed in Section 1.3.2.6). In contrast, apoptosis may result in mycobacterial death. However, the way in which apoptosis is triggered may be crucial. Extracellular ATP (eATP) can trigger macrophage apoptosis via the purinergic receptor, P2z, leading to substantial death of intracellular BCG, whilst apoptosis induced by fas or CD69 ligation failed to affect BCG viability (Molloy et al. 1994; Laochumroonvorapong et al. 1996). Further work is required to demonstrate if eATP-mediated apoptosis represents an important mechanism *in vivo*. However, whatever the mechanism(s) of cell death, one could envisage a dynamic state within the granuloma where repeated cycles of cell death and mycobacterial re-phagocytosis occur; the number of organisms, rate of cell death and degree of macrophage activation all resulting in an outcome of infection control or dissemination.

1.4 CANDIDATE ANTIGENS FOR A SUB-UNIT VACCINE

It has been difficult to identify antigens which induce protective immunity against TB. In part, this may be due to the fact that protection requires a broad immune response with recognition of a large number of antigens and the induction of several crucial effector mechanisms. Studies have suggested that this scenario is likely in the murine TB infection model (Orme et al. 1993a). However, the induction of protective immunity does not have to mimic natural infection. Mounting evidence has identified two groups of proteins which have potential for use in sub-unit vaccine formulation. These include the heat shock proteins (hsp), particularly hsp60, and the CF proteins. Prior to describing individual vaccine candidates, certain methodological issues are discussed, indicating the limitations of ascertaining which proteins may be immunoprotective.

1.4.1 Methodological issues

Studies used to identify immunodominant proteins have been prone to methodological problems. Three such problems are described.

1.4.1.1 *Protective immunity versus T cell reactivity*

In any given assay, induction of T cell responsiveness may not equate with protection. Several studies have measured the responsiveness of murine immune T cells to antigens in terms of proliferation and cytokine production, usually IFN- γ (Kaufmann et al. 1987; Andersen et al. 1991; Huygen et al. 1992). A wide variety of mycobacterial extracts (cell sonicates, PPD etc) and purified protein preparations have been used. A T cell Western assay has also been employed using CF proteins resolved using SDS-PAGE (Andersen and

Heron, 1993). In different laboratories, a large number of protein targets have been identified. Whilst this provides screening for immunodominant proteins, it does not select those which are 'immunoprotective' i.e. those antigens, which upon immunisation, could induce long-lived immune protection. For example, T cells responding to a particular antigen may simply reflect a regulatory function, perhaps in response to inflammation (Orme et al. 1993a). Also, in the mouse model, it has been known for some time that delayed-type hypersensitivity (DTH) does not correlate with protection (Orme and Collins, 1984). This has been demonstrated in humans in a study in Malawi, where marked PPD skin test reactivity (>10 mm induration) of healthy individuals predicted subsequent development of clinical disease rather than protection (Fine et al. 1994).

1.4.1.2 *Kinetics of the T cell response*

There is variation in the type of T cell response occurring at different times after infection. For example, in a murine model, Orme (1992) and colleagues describe the kinetics of emergence and loss of different populations of T cells following MTB infection. They provide evidence that the populations bear different phenotypic markers, recognise different antigens and may perform distinct functions. Thus, identification of a high level of T cell reactivity to a particular antigen two weeks post infection (pi) may not be true for T cells harvested from the same mouse 6 weeks pi.

1.4.1.3 *The form of antigen delivery is crucial*

Much of the work identifying immunodominant antigens has relied on soluble mycobacterial proteins and extracts. This form of antigen delivery has been used for the identification of MTB-specific reactive T cells (Kaufmann et al. 1987; Andersen et al. 1991; Orme et al. 1992; Orme et al. 1993) or the induction of T cell clones (Emmrich et al. 1986; Oftung et al. 1987; Manca et al. 1991). However, the use of 'exogenous' antigen would bias antigen processing in favour of MHC class II presentation and thus skew T cell identification in favour of the CD4⁺ phenotype. This is of crucial importance as there is clear evidence for a protective role performed by MHC class I restricted T cells (Flynn et al. 1992), yet until recently, *in vitro* assays were not designed for their identification, nor can it be assumed that they would recognise the same antigens.

1.4.2 Heat shock protein 60 (hsp60)

The arguments in favour of hsp60 as a candidate immunoprotective antigen are contentious (Kaufmann et al. 1987; Orme et al. 1993; Silva and Lowrie, 1994; Tascon et al. 1996). Early studies demonstrated strong immune responses against hsp60 using immunisation with heat-killed, MTB in adjuvants (reviewed by (Orme et al. 1993a). In one study, hsp 60 reactive-T cells could be readily identified following murine immunisation and limiting dilution analysis demonstrated a frequency of 20-25% reactive cells (Kaufmann et al. 1987). Furthermore, human T cell clones, raised using BCG or MTB, showed strong reactivity to recombinant hsp60 (Emmrich et al. 1986; Oftung et al. 1987).

Critics have argued that immunisation with heat-killed organism would mean that one was immunising with artificially high levels of the hsp60, as autoclaving would induce the protein just prior to cell death. Moreover, they indicate that in mouse models of live MTB infection, the $\alpha\beta$ T cell response to hsp60 was negligible (Huygen et al. 1992). A further argument relates to the kinetics of immune induction. It is known, from adoptive transfer experiments in mice, that protective immunity is induced in the early phase of infection (Orme, 1987), yet in a non-stressful environment, hsp60 is hard to detect (Orme et al. 1993a).

Despite these objections, subsequent mouse protection studies using *M. leprae*-derived hsp60 in a transfected tumour cell line (Silva and Lowrie, 1994) and in DNA vaccine form (Tascon et al. 1996) demonstrated significant protection following challenge with MTB up to 4 weeks post-immunisation. It is intriguing that such a highly conserved molecule, with marked homology to human heat shock protein 65, would induce a protective effect. Of note, the published studies did not ascertain the duration of immunological memory. It is possible that the effects seen might be limited to the initial immune response. Further studies assessing duration of memory and adoptive protection would address this issue.

1.4.3 Secreted proteins

1.4.3.1 T cell reactivity to CF proteins

An increasing body of evidence supports an immunodominant role for MTB secreted proteins (also known as CF or export proteins) (Andersen et al. 1991; Huygen et al. 1992; Orme et al. 1992; Orme et al. 1993; Horwitz et al. 1995). Initial experiments identified secreted proteins as the major targets for T cell proliferation and gamma-interferon (IFN- γ) production using cells harvested from animals immunised with live organism (Andersen et

al. 1991; Orme et al. 1992, Orme et al. 1993). One of the studies demonstrated that the T cells were CD4+ and MHC class II restricted and were only induced if live, but not dead, MTB was used (Andersen et al. 1991). In another study, a strong Th1-type response (5-10 fold increase in interleukin-2 (IL-2) and IFN- γ secretion) was seen in spleen cells from BCG-vaccinated mice when exposed to CF proteins but not to whole BCG or BCG extract (Huygen et al. 1992).

There is additional circumstantial evidence in support of a role for CF proteins inducing protection. These proteins are secreted very early in mycobacterial growth and can be identified as early as the third day of mycobacterial culture (Andersen et al. 1991). It is also known that protective immunity is induced in the early phase of infection and only with live organisms (Rook et al. 1986; Orme, 1987; Orme, 1988). Moreover, only live organisms will induce a significant immune response to secreted antigens. A simple explanation would be that dead organisms do not secrete these proteins resulting in a significant quantitative difference between live and dead bacilli. This would be in contrast to the somatic antigens which are present in similar abundance irrespective of mycobacterial viability (Andersen et al. 1991).

1.4.3.2 Rationale for choice of Antigen 85 complex (Ag85)

Using fractionated CF protein, Andersen and colleagues (1992) demonstrated that a 26-35 kDa fraction of CF proteins was one of two main targets for much of the T cell reactivity. Two proteins were the key targets recognised, Ag85A (also known as P32) (Huygen et al. 1992; Huygen et al. 1994) and Ag85B (Andersen et al. 1992). Two groups have identified Ag85B as being immunodominant (Andersen et al. 1992; Horwitz et al. 1995), although others have not observed such a difference (Dr K. Huygen, personal communication). It is possible that the apparent disparity could result from differences in antigen preparations. The importance of Ag85A as a T cell target had previously been demonstrated in guinea pigs (De et al. 1987), humans (Huygen et al. 1988), and mice (Huygen et al. 1992), although these studies did not compare Ag85A with the other Ag85 proteins or other CF proteins.

Ag85A and 85B constitute two of three proteins collectively known as the Ag 85 complex (Wiker and Harboe, 1992), the third being called Ag85C. They are outer cell wall proteins, but account for a major component of CF protein (Wiker et al. 1986; Wiker et al. 1988; Wiker and Harboe, 1992) with 20-150 times higher concentrations in CF than in cell

sonicate. They are related both at a DNA and protein level and signal sequences have been identified for all three proteins (Content et al. 1991). They bind fibronectin to varying degrees, a potentially important function in macrophage uptake (Abou Zeid et al. 1988; Abou Zeid et al. 1991). Recently, Belisle and colleagues have indicated that Ag85B functions as a mycolyl transferase, suggesting that outer wall cell synthesis may be its key function (Belisle et al. 1995).

1.4.3.3 Ag85 proteins may be recognised by CD8+ T cells

Three studies provide evidence for recognition of Ag85 proteins by CD8+ T cells. In a human pleural TB study comparing T cell immunity in peripheral blood and the pleural space, CD4+ T cell clones were generated using restimulation with purified protein derivative (PPD). Unlike PPD-generated clones from peripheral blood, the 'pleural-space' clones demonstrated limited clonal heterogeneity with over a third of the cells reacting to both a 27-35 kDa band of mycobacterial proteins and also to the purified P32 antigen (85A) (Manca et al. 1991). Of note, these clones could be raised very early in the disease, prior to PPD skin conversion; whilst peripheral blood clones could only be raised after the patient had become PPD skin test positive. From a methodological perspective, stimulating with PPD would select for the CD4+T cell phenotype (exogenous antigen processing), but interestingly, the overall majority of T cells within the pleural space were CD8+positive.

In a another study of pleural TB, human CD8+T cell clones proliferated to a 27-45 kDa band of MTB fractionated protein using a T cell Western assay (Rees et al. 1988). However, the workers did not identify the specific proteins recognised. A further study used Ag 85A to induce human cytotoxic responses against macrophages pulsed with whole MTB (Munk et al. 1994). In this study, T cell phenotyping was not reported although as in the first of these studies, the use of soluble antigen would suggest a predominance of the CD4+phenotype.

1.4.3.4 Why assess all three Ag85 proteins?

The Ag85 complex comprises of genomically and immunologically distinct proteins. The gene sequences for these proteins have been derived for MTB (Borremans et al. 1989; Content et al. 1991) BCG (Matsuo et al. 1988) and other mycobacterial species (Matsuo et al. 1990; Lima et al. 1991). These genes are not clustered together and sequence homology varies between proteins ranging from 70.8-77.5%. This permits coding for unique epitopes

(Content et al. 1991). However, of note, this sequence variation does not occur for the same protein across closely related species (De Wit et al. 1990).

1.4.3.5 *Rationale for choice of ESAT6*

An infection model of human TB in C57BL/6 mice had previously been established which used a parenteral route of inoculation (Andersen et al. 1991). This model was used to assess the memory immune T cell responses, as measured by proliferation and INF- γ production, to fractionated short-term culture filtrate (ST-CF) proteins. This group defined short-term as 1 week of mycobacterial growth in Sauton's medium (Andersen et al. 1992). Very high degrees of reactivity were noted to the low molecular weight protein bands (4-11 kDa). A subsequent study in the same mouse TB model demonstrated that a resting memory cell population was stimulated early in secondary infection with production of large amounts of INF- γ . The molecular target recognised by these T cells was the 6 kDa protein (Andersen and Heron, 1993). Using human T cells obtained from TB patients with either minimal or advanced disease, marked INF- γ production in response to a <10 kDa ST-CF fraction was noted in 6 out of 8 patients with minimal disease, but none with advanced disease (Boesen et al. 1995). This study did not identify discrete proteins in this low molecular weight fraction. These results, taken together, suggest that this protein (plus perhaps other low molecular weight proteins) is an important immunodominant antigen in the immune response to MTB.

The gene for ESAT-6 (designated *esat-6*) has been cloned and sequenced (Andersen and Heron, 1993). The protein, unlike most other CF proteins, has no signal sequence (Sorensen et al. 1995). ESAT-6 was absent from all tested strains of BCG, but present in virulent *M. bovis* (Harboe et al. 1996). In support of this finding, its gene, *esat-6*, was identified in a 9.5 kb region (RD1) deleted from BCG. This region is believed to be the original mutation leading to attenuation of *M. bovis* (Mahairas et al. 1996). The absence of *esat-6* from BCG makes it a potential diagnostic tool to distinguish those vaccinated from those infected with virulent organism.

1.4.3.6 *Animal protection studies using Ag85*

Whole CF in adjuvant has induced protective immunity in mouse (Gough, 1988; Andersen, 1994; Nicholson et al. 1996) and guinea pig (Horwitz et al. 1995) models of TB. In the mouse studies, the protective efficacy was equivalent to vaccination with BCG. In the

guinea pig study, no BCG control was performed. However, this study did include immunising with six purified CF proteins as well as whole CF. Substantial immunity using Ag85B but not other proteins was demonstrated (Horwitz et al. 1995). The type of adjuvant used was important, some adjuvants giving a protective response associated with Th1 T cell stimulation (e.g. incomplete Freund's) and others giving a non-protective Th2 type response (e.g. alum). Unfortunately, alum is the only adjuvant fully licensed for human use.

In a study using DNA vaccination, an Ag85A-encoding construct induced protection to the same order of magnitude as BCG in both TB and BCG models of protection (Horwitz et al. 1995). Unpublished work has also demonstrated equivalent efficacy using an Ag85B construct (Dr K. Huygen, personal communication). In both cases, protection was enhanced by using a eukaryotic signal sequence belonging to human tissue plasminogen activator (tPA) (Horwitz et al. 1995) (Dr K. Huygen, personal communication). The presence of this signal sequence had previously been shown to produce a high level of expression using a transfected cell line system (Chapman et al. 1991).

1.4.4 Summary - Which candidate antigen?

This section has provided evidence in favour of secreted proteins as potential candidate antigens, in particular, the Ag85 complex and ESAT-6. Much of the evidence suggesting the importance of these actively secreted proteins comes from the fact that live and not dead mycobacteria are required for the induction of immunity; whole CF protein/adjuvant preparations are able to confer protection in murine and guinea pig models of TB. T cell reactivity studies provide additional data in favour of Ag85 and ESAT-6. However, somatic antigens, such as hsp60, have also been able to induce immunity. Even more surprising, mycobacterial products, such as the 36 kDa proline-rich protein, thought not to be 'immunodominant' can also confer protection (Tascon et al. 1996). Therefore, it is important to remain circumspect when addressing issues concerning antigen candidature.

1.5 ANTIGEN DELIVERY SYSTEMS FOR SUB-UNIT VACCINATION AGAINST TB

A variety of carriers for candidate TB vaccines have been explored as described above. These include CF proteins given with adjuvant, recombinant bacteria (*Mycobacteria* spp, *Salmonella* spp and *Listeria* spp), 'naked' DNA and recombinant virus (Malin and Young, 1996). So far, available antigen delivery systems have failed to produce a candidate which

consistently performs better than BCG. DNA vaccination looks very promising and clearly represents one useful approach. Safety aspects such as the potential genomic incorporation and mutagenesis have yet to be clarified. Another system which has not been tested extensively for protective efficacy is the use of poxvirus carriers (Lyons et al. 1990; Baumgart et al. 1996).

1.6 RECOMBINANT VACCINIA

1.6.1 Antigen delivery - a need for a sophisticated system

Ideally, an antigen delivery system should have the capacity to deliver one or more antigens (or epitopes) in a form that will induce a 'protective balance' of effector cells, cytokines and antibodies. It would also be beneficial if such a delivery system could co-express immunomodulatory molecules. This would 'fine tune' an immune response in order to maximise protective efficacy. The vector or adjuvant should also be safe and able to deliver antigen to a wide variety of sites in order to permit induction of local as well as systemic immunity.

1.6.2 Why choose a poxvirus to deliver recombinant antigen?

Poxviruses represent one such vehicle. They are cytoplasmic DNA viruses, capable of incorporating a large amount of foreign DNA. Manipulation of the viral genome has been well characterised and construction is relatively straightforward (Mackett, 1995). Numerous vaccinia promoters have been utilised and foreign genes have been inserted in several non-essential loci. In this way, a single construct was able to vaccinate against multiple pathogens (hepatitis B, herpes simplex and influenza viruses) (Perkus et al. 1985). For ease of construction, a large coding region can be inserted into one site permitting the expression of a lengthy polypeptide or, alternatively, a tandem arrangement of multiple genes under different vaccinia promoters. Furthermore, immunomodulatory genes can be included (Rolph and Ramshaw, 1997), permitting the co-expression of cytokines (Carpenter et al. 1994; Leong et al. 1994) or second signal molecules (Rao et al. 1996).

From an immunological perspective, poxviruses enable recombinant antigen processing in the context of both MHC class I and class II pathways (Smith, 1993), which is a likely prerequisite for effective immunity against TB. Other advantages include: vaccine stability and freeze-drying (without need for cold storage), low manufacturing costs and ease of administration.

1.6.3 rVV as a tool to dissect CD8+ T cell responses

rVV represent an excellent tool to assess CD8+ CTL from human or animal origin. Particular features include tropism for a very wide range of cells and excellent processing and presentation of whole antigen in the context of MHC class I (Smith, 1993). This permits mapping of CD8+ T cell epitopes without relying on peptide-prediction algorithms, which may miss biologically important peptides (Sadovnikova et al. 1994).

Factors which must be born in mind when developing constructs for the purpose of CTL assessment include: (i) the need to express protein under an early promoter (MHC class I presentation of some late viral protein is blocked) (Townsend et al. 1988); (ii) CTL may fail to recognise some protein products which are not readily degraded in the cytoplasm (this can be reversed by destabilising the protein by fusing it with ubiquitin) (Townsend et al. 1988); and (iii) recombinant protein under the control of late promoters may not be expressed in all CTL target cells (protein expression should be verified in the target cells used in a CTL assay) (Broder et al. 1994).

rVV derived from wild type strains provide an excellent means for recombinant protein expression in CTL target cells, but are too lytic to be of use as a source of antigen for CTL restimulation. Host-range restricted poxviruses represent an alternative vector for recombinant protein T cell restimulation as many of these viruses are replication-defective in mammalian cells (see Section 1.6.5.2).

1.6.4 Vaccinia - vaccine and vaccine vector

The prototype orthopox virus, vaccinia (VV), holds the unique position of being the only vaccine that has been used to eradicate a global disease. In addition, it has been used as a vector for expressing numerous foreign genes of human, animal, bacterial and viral origin (Tartaglia et al. 1990). Animal vaccination has protected against a large number of infections including influenza, herpes simplex, hepatitis B and rabies (Brochier et al. 1990; Tartaglia et al. 1990). Several MTB and *M. leprae* rVV have been made (Lyons et al. 1990; Baumgart et al. 1996) and murine immunisation with a rVV construct expressing the *M. leprae* 18 kDa protein was able to confer protection against challenge with BCG (Baumgart et al. 1996).

1.6.5 A need for a highly attenuated host-range restricted strain

1.6.5.1 *Problems with wild-type vaccinia*

The smallpox eradication campaign highlighted specific safety issues concerning the use of vaccinia. Rare but serious post-vaccination adverse effects were associated with some of the vaccine strains in otherwise healthy vaccinees. The worst of these was encephalitis. Less severe, but a nuisance nevertheless, was skin involvement in those individuals suffering from eczema (Smith, 1993). The greatest problem of all is the possibility of severe, disseminated viraemia in the immunocompromised host, making the use of a wild type (wt) recombinant vaccine unacceptable. This is a significant problem considering that many who would benefit from vaccination against TB would be infected with HIV (Bloom and Murray, 1992).

Another issue relating to wt VV is the fact that it encodes many proteins which permit evasion of host immunity (Smith, 1993). Theoretically, this could limit its use as a vaccine vehicle by down-regulating the immune response. One of the key immune evasion strategies includes secretion of virally-encoded soluble cytokine receptor homologues. These receptors compete for their cognate cytokines at the expense of the cellular counterparts. Examples include: IL-1 β receptor (B15R) (Alcami and Smith, 1992); IFN- γ receptor (B8R) (Alcami and Smith, 1995); INF- α/β receptor, (B18R) (Symons et al. 1995) and TNF- α receptor. Given that these genes have evolved in order to evade immunity, it is not surprising that, in some cases, deletion results in virus attenuation (Symons et al. 1995).

1.6.5.2 *Non-replicating poxvirus*

Non-replicating poxviruses represent an attractive alternative to wt VV. They offer the advantage of a live virus vehicle which permits rapid cell attachment to a wide variety of cells, invasion and correct antigen processing; but not the disadvantage of unwanted dissemination, either within the vaccinee, from the vaccinee to non-vaccinated contacts or to the environment. Avian poxvirus recombinants, which fail to replicate in mammalian cells, still correctly process and present foreign antigens to mammals. For example, a canarypox recombinant protectively immunised cats and dogs against rabies (Cadoz et al. 1992) and elicited a neutralizing antibody response in humans (Taylor et al. 1991). An alternative non-replicating poxvirus is modified vaccinia virus Ankara (MVA); this is an attenuated derivative of one of the smallpox vaccine strains, vaccinia Ankara (Sutter and Moss, 1992). This particular deletion mutant represents, perhaps, the best of all the potential poxvirus vaccine vehicles. The reasons for this are outlined below.

1.6.5.3 Recombinant MVA

During the course of the smallpox eradication campaign several highly attenuated strains of vaccinia were developed. One of these, MVA, was derived following over 570 passages in chick embryo fibroblasts. This resulted in 6 major deletions with the loss of 15% of its genome, which rendered it unable to grow in mammalian cell lines (Meyer et al. 1991). Despite this host restriction, MVA was successfully used to immunise more than 120 000 humans against smallpox without significant side-effects. Importantly, it was also found to be avirulent in a wide variety of immunosuppressed animal hosts (Werner et al. 1980). Recombinant MVA (rMVA) expressing influenza haemagglutinin and nucleoprotein showed induction of specific antibody and CTL responses and protection against a lethal challenge from influenza virus (Sutter et al. 1994). Remarkably, as low as 10^4 plaque-forming units (pfu) were able to protect the majority of mice against a 100 LD₅₀ challenge.

Further theoretical advantages of MVA include an ideal profile with the loss of functional genes encoding soluble cytokine receptors IFN- γ R and IFN- α/β R but not IL-1 β R (Blanchard et al. 1997). Both IFN receptors down-regulate immune responsiveness and are associated with enhanced virulence and persistence of virus. Indeed, rVV expressing IFN- γ enhances cell-mediated immunity and viral clearance. Although IL-1 β R is still expressed, this latter cytokine receptor has been associated with a reduction in virulence in infected mice in an intranasal challenge model (Alcami and Smith, 1995). Consistent with this finding, the safer human smallpox vaccine strains, such as Lister and Wyeth, express IL-1 β R (Smith, 1993). By contrast, higher levels of post-vaccinal complications were noted in those strains where the receptor was absent or only expressed at low levels.

1.6.6 Poxvirus recombinants and co-expression of cytokines

Several cytokines have been successfully expressed in both wt VV (Carpenter et al. 1994) and avipoxvirus (Leong et al. 1994). For example, in one study, rVV-IFN- γ caused up-regulation of cellular IFN- γ and increased viral clearance (Carpenter et al. 1994). Also, the non-replicating fowlpox virus (FPV) has been constructed to co-express influenza virus haemagglutinin and IFN- γ . CTL activity was high for rFPV both with and without the cytokine, but the expression of IFN- γ resulted in total abrogation of a humoral response (Leong et al. 1994). From a safety perspective, this cytokine-encoding rFPV was non-pathogenic in severe combined immunodeficient (SCID) mice.

1.6.7 Use of the T7 promoter

The bacteriophage T7 promoter has been used in a variety of prokaryotic and eukaryotic expression systems. Due to its very high processivity and exclusive requirement for T7 RNA polymerase, it has been useful both for cell-free *in vitro* transcription/translation and intracellular expression using a rVV expressing T7 polymerase.

1.6.7.1 *In vitro* transcription/translation using the T7 system

Starting with circular plasmid DNA containing the gene of interest under the control of the T7 promoter, it is possible to obtain a signal from metabolically-labelled protein products within a short period of time using an entirely cell free system (Schenborn, 1995). Transcription requires T7 polymerase, the plasmid template and nucleotides plus buffer. Translation can then be performed, either as a separate reaction, or within the same reaction mix. This step requires an appropriately prepared membrane-free, cell lysate containing the essential translational apparatus to which amino acids are added. One or more of these should contain a radionuclide label such as ^{35}S , thus permitting metabolic labelling of protein and autoradiographic signal detection. Using a commercial kit, a coupled transcription/translation reaction can be performed and protein signal detected within 6 hours. In addition, by adding microsomal membranes to the *in vitro* reaction, the effects of post-translational glycosylation can be studied (Schenborn, 1995).

1.6.7.2 *The vaccinia/T7 hybrid system*

This system permits very high level transient expression of foreign genes. The system entails *in vitro* cellular infection using a rVV expressing T7 polymerase (vTF7-3), plus one or other of: (i) a second rVV coinfection; or (ii) a plasmid transfection, either of which must contain the foreign gene under the control of the T7 promoter (Mackett, 1995). For example, the rVV/plasmid system demonstrated a 400-600 fold increase in reporter gene activity as compared with other standard mammalian transient expression systems of the day (Fuerst et al. 1986). Using the rVV coinfection system with a *lacZ* reporter, approximately 30% of the total cellular RNA was T7 specified at 24 hours post-infection. However, over 90% was uncapped and poorly translated (Fuerst and Moss, 1989). This problem was overcome by including a 5' untranslated leader sequence within the transcript. This was derived from encephalomyocarditis virus (EMCV) which, like other picornaviruses, contains an internal ribosomal entry site (IRES). This region permits highly efficient cap-independent translation

(Vennema et al. 1991). In one study using the vaccinia/T7 hybrid system, the foreign gene product accounted for over 10% of the total cellular protein.

1.7 SUMMARY

This chapter has argued that an alternative vaccine to BCG is urgently required, particularly as the current vaccine fails to protect against the transmissible adult pulmonary disease. From the perspective of safety and quality-control, a rationally designed sub-unit vaccine would be particularly desirable. However, its development requires knowledge of what constitutes an effective immune response. Human immunity to MTB is poorly understood, but there is increasing evidence in favour of a contribution from CD8⁺ as well as CD4⁺ T cells. The precise role for this CD8⁺ T cell contribution is not known and evidence from knockout mice studies suggests that their importance may not be due to a cytolytic function. An alternative potentially important function for CD8⁺ T cells could be the provision of additional IFN- γ , particularly where CD4⁺ T cell recognition may be compromised by down-regulation of MHC class II molecules in MTB-infected macrophages.

MTB secreted proteins represent likely candidate antigens for a sub-unit vaccine. Two antigenic groups, ESAT-6 (plus perhaps other low molecular weight secreted proteins) and the Ag85 proteins, are particularly strong candidates. In terms of the vehicle for antigen delivery, poxviruses show several highly desirable properties: (i) MHC class I as well as class II antigen presentation; (ii) vaccine potential, using host-range restricted, highly attenuated strains; and (iii) as a tool to dissect human CD8⁺ T cell responses.

In order to utilise the benefits of a vaccinia expression system in conjunction with key immunodominant MTB proteins, this study aimed to produce a panel of rVV expressing ESAT-6 and Ag85, with the intention of using the rVV both as tools to assess human CD8⁺ T cell function and potential vaccines in animal models of TB.

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2.1 WATER AND SOLUTIONS

Distilled water was used to make up media and buffers. Autoclaved double deionized water (Millipore, Watford, UK) was used for all molecular biology reactions. All solutions used in RNA work were made with autoclaved, diethyl pyrocarbonate (DEPC) treated water. All standard salt solutions and buffers were made up as described elsewhere (Sambrook et al. 1989).

2.2 BACTERIAL CULTURE MEDIA AND BROTHS

Media and broths were prepared according to the manufacturer's instructions or as described below. Luria broth (LB) (Life Technologies, Paisley, UK) and LB agar containing ampicillin (Sigma, Poole, UK) at 50 µg/ml (stock 50 mg/ml stored at -20°C) were used for culturing transformed *Escherichia coli*. LB broth plus tetracycline (Sigma) at 50 µg/ml (stock 5 mg/ml in 50% v/v ethanol stored at -20°C) was used for M13 bacteriophage growth.

Mycobacteria broth culture was performed using either Middlebrook 7H9 (Difco, West Molesey, UK) supplemented with 10% w/v ADC supplement (Appendix A) or 10 % w/v oleic acid-albumin-dextrose-catalase supplement (OADC, Becton Dickinson, Cockeysville, MD, USA) or a minimal synthetic Sauton's medium (Appendix A). Mycobacteria solid phase media were either Lowenstein-Jenson slopes or Middlebrook 7H11 (Difco) supplemented with OADC.

2.3 BACTERIA

2.3.1 *Escherichia coli* strains

Competent *E. coli* strains were used for transformation and propagation of plasmids using Epicurian Coli supercompetent SCS1 cells, (Stratagene, Cambridge, UK) and M13 bacteriophage using Epicurian Coli supercompetent XL1-Blue (Stratagene). All bacteria were stored at -80°C.

2.3.2 Bacterial transformation

Bacterial transformation was performed in accordance with the protocol from Stratagene. In order to enhance efficiency, the system was optimized by using a range of: (i) ligation mix volumes (1-8 µl); (ii) concentrations of vector DNA (0.5-50 ng); and (iii) vector:insert molar ratios (1:5-1:90). Transformants were identified as discrete colonies grown on LB



plates containing 50 µg/ml of ampicillin. These were picked and grown up overnight in LB/ampicillin broth prior to plasmid purification.

Purified plasmids were archived and stored at -20°C. In addition, 1 ml aliquots of broth were catalogued and stored in cryotubes (Greiner, Dursley, Glos, UK) in 30% v/v glycerol, at -80°C.

2.3.3 Mycobacteria culture

2.3.3.1 Preparation of stock culture

Mycobacteria stock for subsequent culture was grown on Lowenstein-Jenson slopes and transferred to Middlebrook 7H9/OADC liquid medium, harvested during log phase growth and stored in 1 ml aliquots containing 30% v/v glycerol prior to use. Strains included: BCG (Glaxo strain) derived from freeze-dried stock (Evans Medical, Leatherhead, UK), *M. marinum* (MM) derived from freeze-dried stock (ATCC 11564, NCTC 10011, 16/9/69) and MTB H37Rv derived from the local laboratory culture which was originally provided by Dr M.J. Colston at NIMR, London.

BCG stock was also grown for CTL work. This was prepared as above but resuspended in RPMI. In this study, the BCG/RPMI stock was kindly donated by Dr J. Turner.

2.3.3.2 Preparation of early culture filtrate protein

Three mycobacteria strains were grown in Middlebrook 7H9/OADC for 7-14 days (MM 7 days, BCG and MTB and 14 days). Additional experiments included growing MM in both Middlebrook 7H9/ADC and Sauton's medium. Cells were pelleted and the supernatant obtained and sterile filtered using a 50 ml syringe and a 0.2 µm filter (Nalgene, Rotherwas, Hereford, UK). For safety reasons, 5 ml aliquots were filtered for the MTB culture.

The culture filtrate was then concentrated using a Centricon 3 membrane filter which retains proteins greater than 3 kDa in size (Amicon, Stonehouse, Glos, UK). Filtration was achieved by spinning the Centricon tubes at 6000 rpm (3000 g) in a Sorvall RC-5 Superspeed Refrigerated Centrifuge using the SS-34 fixed angle rotor. The protein content was measured using the Bradford method (Maizels et al. 1991) and aliquots were frozen at -70°C.

2.4 PLASMIDS

2.4.1 Vaccinia insertion plasmids

Three vaccinia insertion plasmids were used for construction of rVV: (i) pSC11 (Figure 2.1a) provided by Dr T Blanchard, LSHTM (previously obtained from Dr H Stauss, ICRF, London) (Chakrabarti S et al. 1985); (ii) p1108 (Figure 2.2a) provided by Dr M Mackett, CRC, Manchester; and (iii) pWR510 provided by Prof J Mullins, University of Washington, Seattle (Figure 2.3a) (Wang and Mullins, 1995).

2.4.2 Plasmid clones containing mycobacterial genes

Clones containing the genes that encode MTB Ag85 complex and the 6 kDa secreted protein, ESAT-6 had been previously cloned into bacterial plasmids (Borremans et al. 1989; Content et al. 1991; Sorensen et al. 1995). The clones were donated by other laboratories and were used as PCR templates for the construction of the vaccinia insertion plasmids required for rVV development. The genes encoding proteins of the Ag85 complex, *85a*, *85b* and *85c*, were provided by J Content, Pasteur Institute, Brussels. The genes had been subcloned into the BlueScribe-M13+ vector (Stratagene) and were designated: pBS-85A, pBS-85B and pBS-85C. The *esat-6* gene had been subcloned into pBlueScript II SK+ (Stratagene) to make the clone, pAA249. Genes *85a*, *85c* and *esat-6* were originally derived from the Rick Young λ gt11 gene library (Young et al. 1985). *85b* was derived from BCG genome (Dr J. Content, personal communication). However, it has identical sequence homology to MTB at the amino acid level (Content et al. 1991). Both the plasmids with *85a* and *85c* are the property of Innogenetics, Ghent, Belgium. A Memorandum of Agreement concerning commercial application of the derivatives was signed prior to their release.

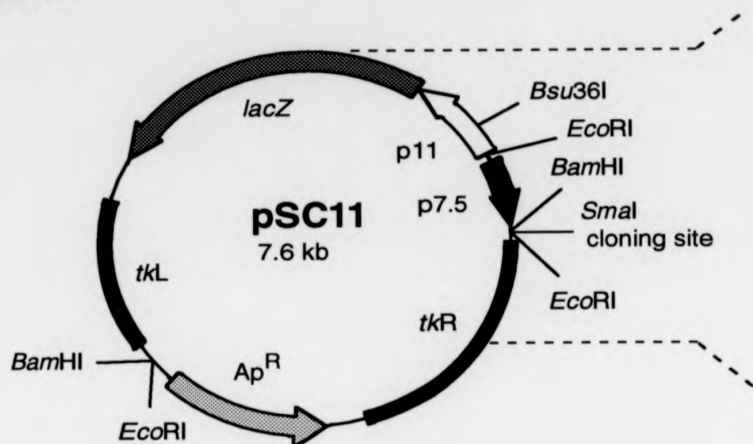
2.4.3 Additional vectors

Sequencing vectors, M13-mp18 and -mp19, were obtained from Sigma.

2.5 PRIMERS AND OLIGONUCLEOTIDES

Primers were obtained from three sources: (i) Oswel DNA Service, Edinburgh, UK (pSC11-ESAT-6 cloning primers and tPA oligonucleotides); (ii) MWG Biotech, Ebersberg, Germany (primers 1 to 20); and (iii) Life Technologies (all other primers unless otherwise stated). The oligonucleotides were HPLC purified. However, none of the primers were HPLC purified. Individual primers were stored as 20 μ M stock solutions at -20°C.

a pSC11



b pSC11-ESAT-6 (detail)

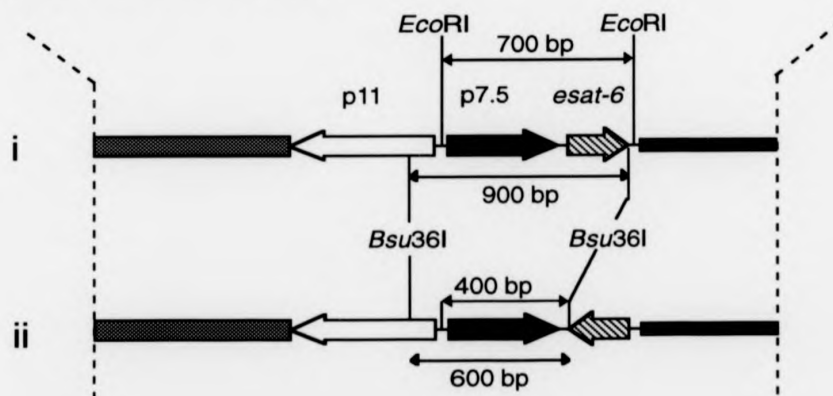


Figure 2.1 pSC11 and pSC11-ESAT-6

a) A map of the vaccinia insertion plasmid pSC11 is shown. This demonstrates the essential elements for rVV construction including: a unique *Sma*I cloning site for insertion of foreign genes under the vaccinia p7.5 promoter; the selectable marker gene, *lacZ*, under the vaccinia p11 promoter, for blue/white selection; and flanking *thymidine kinase* (*tkL* and *tkR*) sequences required for homologous recombination. **b)** The *esat-6* gene was cloned into the *Sma*I site using T:A cloning which created a second *Bsu*36I site at the 3' end of the ORF. Thus, the correct orientation could be determined from a single *Bsu*36I digestion. The results of this digest would produce: **i** a 900 bp fragment indicating correct orientation or **ii** a 600 bp fragment indicating incorrect orientation.

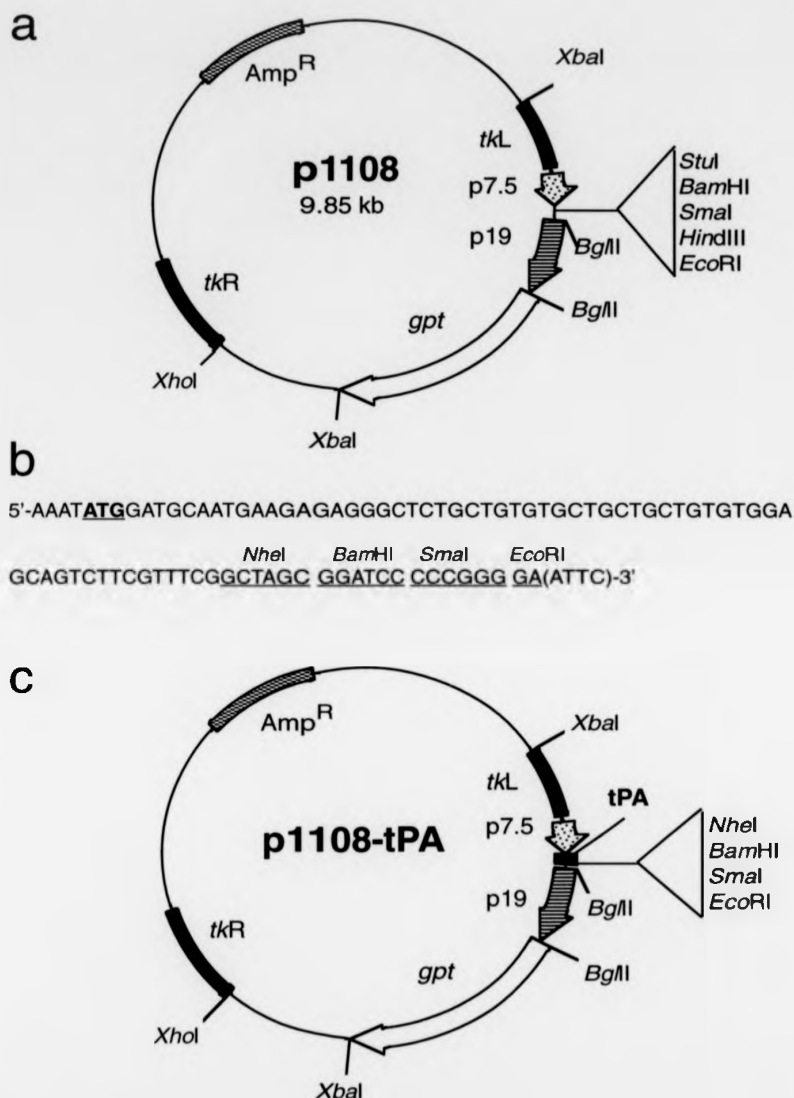


Figure 2.2 Cloning the tPA signal sequence into p1108

a) p1108 is derived from pBR328 and includes the essential elements for vaccinia homologous recombination: flanking thymidine kinase sequences (*tkL* and *tkR*), a multiple cloning site (MCS) downstream from the early/late p7.5 promoter for insertion of foreign DNA, and the selectable marker, *guanine phosphoribosyl transferase* gene (*gpt*), under control of the 19 kDa promoter. Amp^R=ampicillin resistance. **b)** tPA sense strand (antisense) includes an additional 4 nucleotide *EcoRI* overhang. The hybridised oligonucleotide pair encodes the 21 amino acid tPA signal sequence and a MCS. **c)** p1108-tPA was constructed by cloning the tPA blunt/*EcoRI* fragment into the *BamHI* cut, Klenow-filled, *EcoRI* cut vector.

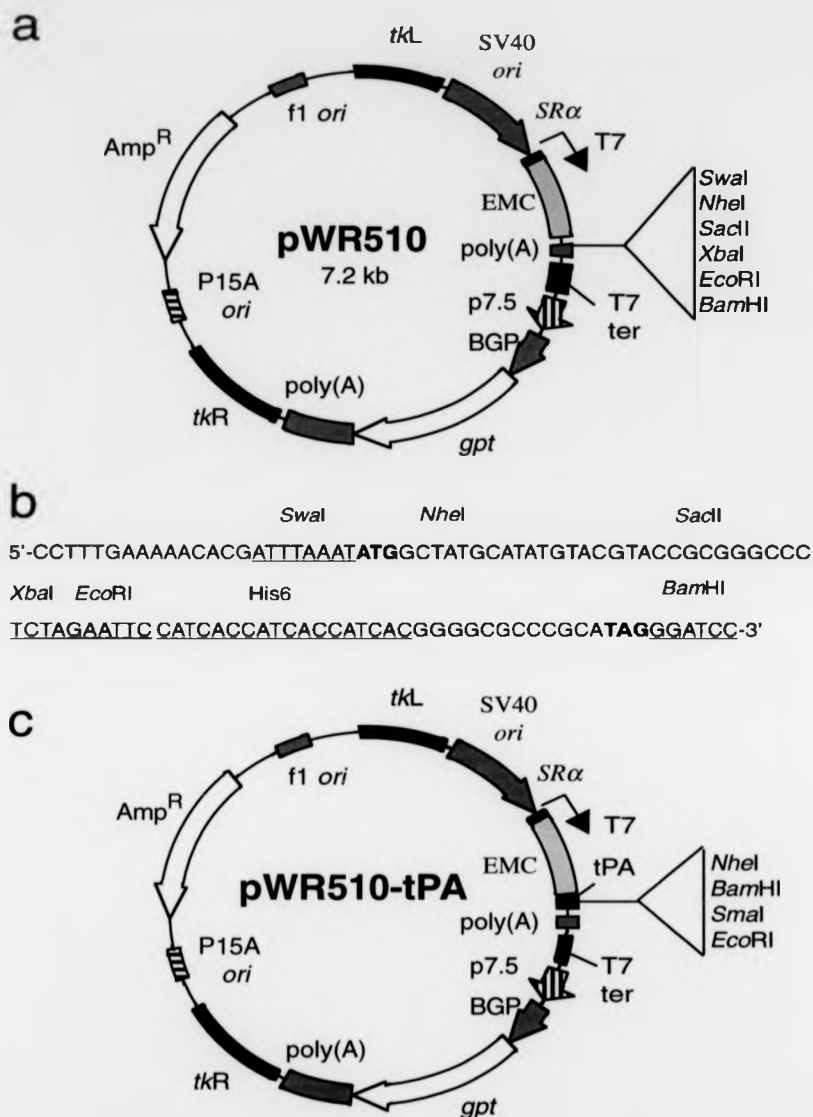


Figure 2.3 Cloning the tPA signal sequence into pWR510

a) Map of pWR510 showing the essential elements for construction of rVV: the components required for cloning and propagation in *E. coli* (*f1 ori*, *Amp^R* and *P15A ori*) and an expression cassette required for homologous recombination with VV (*SRα/T7* promoter, EMC element, poly(A) and T7 terminator (*ter*), the selectable marker gene *gpt*, and *tkL* and *tkR* sequences). **b)** The nucleotide sequence of pWR510 surrounding the start codon, unique cloning sites and His6 tag. **c)** pWR510-tPA was constructed by cloning the tPA blunt/*EcoRI* fragment into the *Swal/EcoRI* cut vector.

2.5.1 Primer nomenclature

This was defined according to an 8 character code (see Figure 2.4). Primer pairs were coded by the 2 digit unique identifier e.g. 01/02.

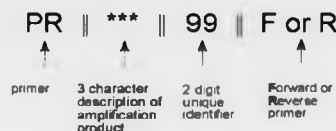


Figure 2.4 Primer nomenclature

2.5.2 Primer design

Primers were designed using a genetic software package (Lasergene, DNASTAR Inc. USA). Optimal primers were identified by using the primer select programme. The software permitted subsequent manual manipulation in order to allow addition and/or substitution of non-complementary nucleotides required for cloning (see below).

2.5.3 Cloning primers


Cloning primers were selected to amplify only the region encoding the mature secreted protein (entire open reading frame (ORF) for *esat-6* which lacks a signal sequence) or as close as possible to this. Thus, in the case of the Ag85 proteins, primers were designed to include a start codon just before the first residue of the mature protein, phenylalanine (Phe). All cloning primers are listed in Table 2.1. The table does not include the first primer pair used for cloning *esat-6* into pSC11 (see Section 2.7.3.1).

The computer programme-generated primers were manipulated to include (from the 5' end): (i) two nucleotides of either dGTP or dCTP for both strong 5' binding and the requirement of an overhang required for endonuclease digestion, (ii) a selected restriction site (either *NheI*, *XbaI* or *BamHI* in the upper primer and *EcoRI* in lower primer) in accordance with the cloning site of the plasmid, (iii) an ATG translation initiation codon (if absent in the vector or amplified coding sequence), and (iv) at least 11 nucleotides of template sequence homology. The programme gave a ranking for each primer pair including the primers modified for cloning purposes. Where a selected primer was predicted to perform badly, an alternative primer, upstream from the

Name	Sequence	Size	Tm (°C)	Gene	Plasmid	Site	Notes [†]
PR85A01R	5'-CGTCTAGATTTTCCCGCCGGGCTT-3'	25	57.5	85a	pWR510	XbaI	start codon at Phe
PR85A02R	5'-CGGAATTCGCGCCCTGGGCGCGG-3'	25	65.7	85a	pWR510	EcoRI	no stop codon
PR85A03F	5'-GCGGATCCATGGCGGGGCATTTTC-3'	25	59.1	85a [‡]	p1108	BamHI	Phe preceded by Ala Gly Ala
PR85A04R	5'-GCGAATTCAGCTAGGGACGGGATT T-3'	25	54.2	85a [‡]	p1108	EcoRI	55 bp downstream from the stop codon
PR85B05F	5'-CGTCTAGATTCTCCCGCCGGGCT-3'	25	60.8	85b*	pWR510	XbaI	start codon at Phe
PR85B06R	5'-CCGAATTCGCGCGCTAACGAATC-3'	25	57.5	85b*	pWR510	EcoRI	error in primer - need EcoRI to be in frame for His tag
PR85B07F	5'-CCGGATCCATGTTCTCCCGCCG-3'	23	59.1	85b*	p1108	BamHI	start codon at Phe
PR85B08R	5'-GGGAATTCGCGTTGATCCCGTCAG-3'	25	55.9	85b*	p1108	EcoRI	native stop codon and 4 codons
PR85C09F	5'-CGTCTAGATTCTCTAGGCCCGGTCT-3'	25	55.9	85c	pWR510	XbaI	start codon at Phe
PR85C10R	5'-GGGAATTCACAGCGGCCGCGCA-3'	24	60.8	85c	pWR510	EcoRI	mutates stop codon only TGA to TGT
PR85C11F	5'-CCGGATCCATGTTCTCTAGGCCCG-3'	24	57.4	85c*	p1108	BamHI	start codon at Phe
PR85C12R	5'-GCGAATTCGCTGGCTCAGCGCG-3'	22	57.2	85c*	both	EcoRI	pWR510 and 1108, stop codon present +3 bp
PR85A13F	5'-GCGCTAGCACCGCGGGGCATTTTC-3'	25	60.8	85a	pWR510	NheI	Phe preceded by Thr Ala Gly Ala
PR85A14F	5'-CCGGATCCATGTTTTCCCGCCGGG-3'	25	60.8	85a	p1108	BamHI	start codon at Phe
PR85A15R	5'-GGGAATTCCTAGCGCCCTGGGGC-3'	24	60.8	85a	pWR510	EcoRI	stop codon only
PR85B16F	5'-CGGCTAGCTTCTCCCGCCGGGGCT-3'	25	64.1	85b	pWR510	NheI	start codon at Phe
PRESA17F	5'-CGGCTAGCGACAGAGCAGCAGTGG-3'	24	59.1	esat-6	pWR510	NheI	error in primer - extra G at position 9
PRESA18R	5'-GCGAATTCATATGCGAATCCAG-3'	25	52.6	esat-6	pWR510	EcoRI	mutates stop codon to Tyr, in frame
PRESA19F	5'-GCGGATCCATGACAGAGCAGCAGTG-3'	25	57.5	esat-6	p1108	BamHI	amplifies ORF (no signal sequence for ESAT-6)
PRESA20R	5'-GCGAATTCCTATGCGAATCCAG-3'	25	54.2	esat-6	p1108	EcoRI	amplifies ORF
PR85C21F	5'-CGGCTAGCTTCTCTAGGCCCGGTCT-3'	25	59.1	85c	pWR510	NheI	start codon at Phe
PR85B22R	5'-CGGAATTCAGCCGGCGCCTAACG-3'	25	60.8	85b	pWR510	EcoRI	mutates stop codon to Tyr, in frame

Table 2.1 Cloning primers

[†]=description of primers with respect to: translational initiation (Phe residue at start of mature protein, upstream from mature protein or at ORF), termination of translation and errors in primer design (see Section 2.5.3), [‡]=*Taq* polymerase used for this pair, *=PCR annealing temperature of 55°C.

 = primers used in p1108 and pWR510 constructs.

start of the mature protein coding sequence (starting with Phe) was also purchased (see notes section in Table 2.1).

2.5.3.1 *pSC11-ESAT-6 primer pair*

The initial cloning experiment included two 20 nucleotide-length (mer) sequences. The primers were designed to amplify *esat-6* prior to T:A cloning into pSC11 - see Figure 2.5 and Section 2.10). The forward primer amplified from the start of the native start codon of the ORF (5'-ATGACAGAGCAGCAGTGGAA-3'). The reverse primer included the stop codon and an additional four non-complementary nucleotides encoding part of a *Bsu36I* site (5'-AAGGCTATGCGAACATCCCA-3'); the remaining T-vector sequence completing the site following successful cloning.

2.5.3.2 *p1108 and pWR510 primers*

Each primer pair was designed to amplify the coding sequence for the mature protein only with exclusion of the mycobacterial signal sequence (Content et al. 1991) (not applicable for *esat-6* which has no signal sequence). Forward primers included a *BamHI* site and a start codon for p1108 and either a *NheI* or *XbaI* site, but no start codon, for pWR510 (Table 2.1). The reverse primer included an *EcoRI* site for all amplification products. For pWR510 reverse primers, the stop codon was removed to allow a C-terminus six residue histidine fusion (His6 tag) (Figure 2.3) for recombinant protein purification using nickel affinity chromatography (Wang and Mullins, 1995). Thus, directional cloning could be performed using PCR restriction enzyme cut products into *BamHI/EcoRI* cut p1108 (Figure 2.2) and *NheI/EcoRI* cut pWR510 (Figure 2.3).

2.5.4 Additional primers used for sequencing and PCR verification

2.5.4.1 *Primers for sequencing pSC11-ESAT-6 and M13 subclones*

External primers flanking the pSC11 cloning site were used in double stranded sequencing of recombinant pSC11-ESAT-6. These were kindly provided by Dr T Blanchard (see Table 2.2). The M13 forward primer (Sigma) was used for single stranded sequencing of M13-ESAT-6 recombinants (mp18 and mp19).

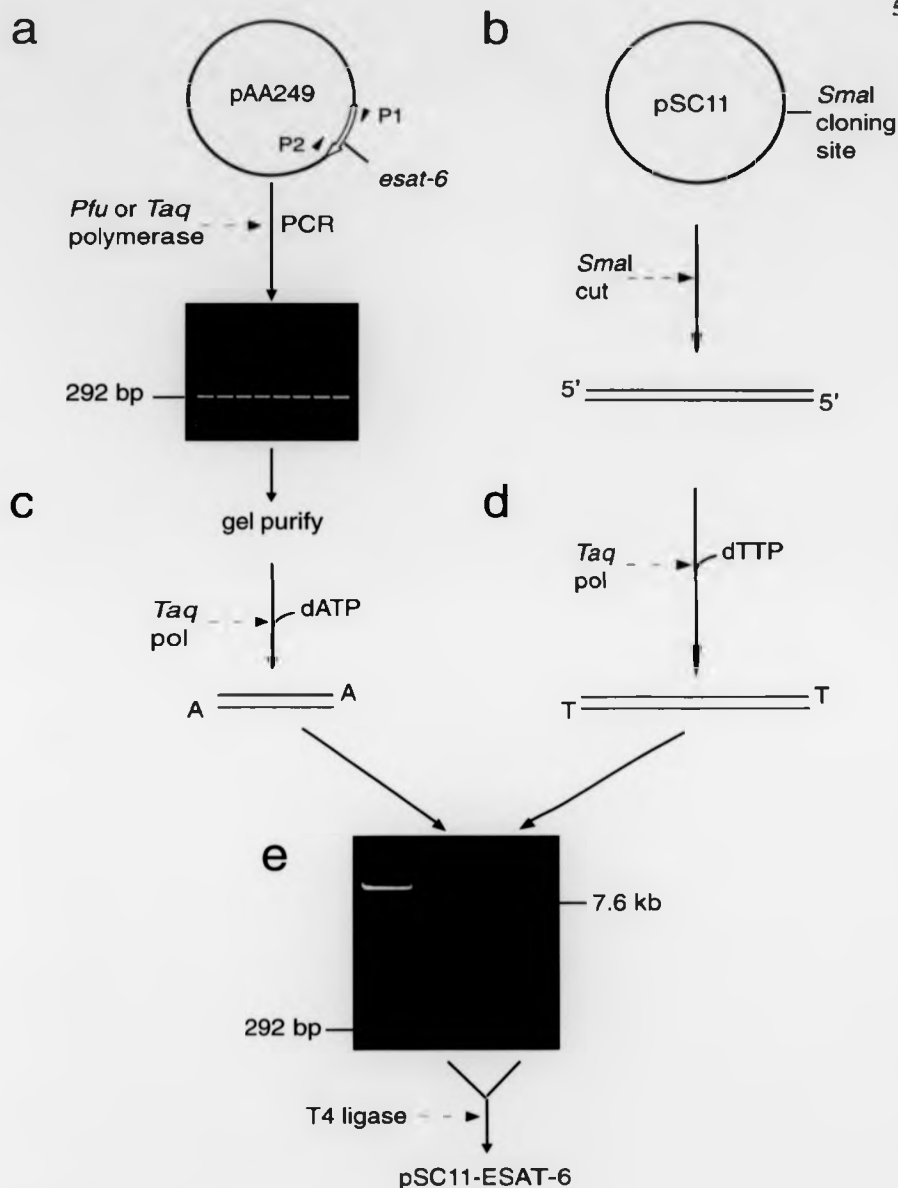


Figure 2.5 T:A cloning of *esat-6* into pSC11

a) An insert carrying *esat-6*, suitable for cloning into the vaccinia insertion plasmid, pSC11, was obtained by PCR amplification from the plasmid pAA249 using primers P1 and P2. b) pSC11 was linearised using *Sma*I restriction digestion. c) The *esat-6* amplification product was gel-purified. The *Pfu* product was tailed with a single dATP (A' tailing was not required for *Taq*-generated products). d) The *Sma*I cut pSC11 was tailed with a single dTTP. e) A'-tailed *esat-6* and T'-tailed pSC11 were ligated to form pSC11-ESAT-6

Name	Sequence	Size (mer)	Region recognized	PCR product size (bp)
PR51023F*	5'-CATGCTTTACATGTGTTTAGTCG-3'	23	pWR510 sense	184
PR51024R*	5'-GTAACCGTTAACGGATCCCTATGC-3'	24	pWR510 sense	
PRtPA25F*	5'-AAATATGGATGCAATGAAGAGA-3'	22	tPA sense	79
PRtPA26R*	5'-GGATCCGCTAGCCGAAACGAA-3'	21	tPA antisense	
PR10827F*	5'-TATTCTAATTTATTGCACGGTAAG-3'	24	p1108 sense	63
PR10840R*	5'-CGCGGGCCCCGAGATCTTAGTTAG-3'	23	p1108 antisense	
PRTK328F	5'-AATTAGACGAGTTAGACG-3'	18	tkL	not known
PRTK4294	5'-ATCTCGGTTTCCTCACCC-3'	18	tkR	
PR85B30F	5'-ATCAACACCCCGGCGTTCGAGTG-3'	23	85A and 85B	n/a
PR85C31R	5'-CGGCAGCTCGCTGGTCAGGA-3'	20	85A and 85B	n/a
PR85C34R	5'-CCGCGTTGCCTGTCGGGG-3'	19	85C	n/a
PR85C35F	5'-CTGGAAGCGCAACGACC-3'	17	85C	n/a
PRPSC42F*	5'-AAACTGATCACTAATTCCAAACC-3'	23	pSC11 sense	n/a
PRPSC43R*	5'-GTTTGCCATACGCTCAC-3'	17	pSC11 antisense	n/a

Table 2.2 Primers used for PCR verification of rVV and sequencing of plasmids

*=primer pairs used for verification of rVV gene integration. All primers were used for sequencing except for PR51024R and PRtPA26R.

2.5.4.2 Primers for p1108, pWR510 and their tPA derivatives

A variety of primers were designed for both sequencing of recombinant insertion plasmids to confirm fidelity of cloning and to provide verification for successful rVV construction. These are listed in Table 2.2. Primer pair 28/29 (TK primers) was given by T. Blanchard, Oxford. Primers 30-33 were given by Dr J. Content, Brussels.

2.6 ENZYMES, KITS, RADIONUCLIDES AND MISCELLANEOUS REAGENTS

All reactions were carried out according to the manufacturer's instructions and reagents were stored at -20°C unless otherwise stated.

2.6.1 DNA restriction enzymes

Enzymes and buffers were purchased from Promega, Southampton, UK unless otherwise stated. The following restriction enzymes were used: *Bam*HI, *Bsu*36I, *Eco*RI, *Hinc*II, *Hind*III, *Nhe*I (Stratagene), *Pst*I, *Pvu*I (Stratagene), *Sac*II, *Sma*I, *Swa*I (Boehringer Mannheim, Lewes, UK) and *Xba*I.

2.6.2 DNA modifying enzymes and related reagents

Individual modification enzymes included: (i) T4 ligase (Promega); (ii) DNA polymerase I Large (Klenow) Fragment (Promega); PCR DNA polymerases - *Taq* (with storage buffer B, Promega) and cloned *Pfu* (Stratagene); (iii) RNase-free DNase (Deoxyribonuclease I, Amplification Grade, Life Technologies); (iv) RNasin ribonuclease inhibitor (Promega); and (v) 100 mM deoxynucleotide triphosphates (dNTPs) (Promega).

2.6.3 Molecular biology kits

- 1) Manual sequencing was performed using the Sequenase Version 2.0 DNA Sequencing Kit (USB, Life Science).
- 2) Cycle sequencing was performed using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase FS (Perkin Elmer, Foster City, CA, USA).
- 3) Reverse transcriptase PCR (RT-PCR) reagents were obtained from Gibco BRL SuperScript Preamplification System for First Strand cDNA Synthesis kit (Life Technologies).
- 4) *In vitro* coupled transcription/translation was performed using the TNT T7 Coupled Reticulocyte Lysate kit (Promega) and stored at -70°C.
- 5) Immunoblotting was performed using the Enhanced Chemiluminescence (ECL) Western blotting Detection system (Life Sciences, Little Chalfont, Bucks., UK) and stored at 4°C.

2.6.4 Radionuclides

Three radionuclides were used in this study: (i) ³⁵S-dATP stored in a 100 µl volume, at 4°C, with an activity 10 mCi/ml on day 0; (ii) ³⁵S-methionine stored in 5 µl aliquots, at -80°C, with an activity 15 mCi/ml on day 0; and (iii) ⁵¹Cr stored in a 1 ml volume, at 4°C, with an

activity range of 1-5 $\mu\text{Ci/ml}$ depending on usage. Radionuclides were purchased from Life Sciences, Little Chalfont, UK and ICN, Thame, UK.

2.7 AGAROSE GEL ELECTROPHORESIS AND DOCUMENTATION

2.7.1 Agarose, running and loading buffers

Electrophoresis grade Ultrapure Agarose was obtained from Life Technologies. For most purposes, 1% w/v gels were poured shortly before use and included 0.02% w/v ethidium bromide (BDH Electran, Merck, Poole, UK). Occasionally, 0.7-3% w/v gels were poured to improve size resolution. Initially, Ultrapure LMP (low melting point) agarose (Life Technologies) was used in DNA purification (pSC11-ESAT-6 cloning only). TAE (Sambrook et al. 1989) was used as the gel tank buffer. When a longer electrophoresis running time was required, ethidium bromide was added to the buffer in order to maintain uniform gel staining. A commercial loading buffer (Promega) containing three colour dyes was initially used, but it was noted that the presence of the dye could obscure a band running at the same speed. As alternatives, 0.25% w/v xylene or 0.25% w/v bromophenol blue in 30% v/v glycerol were prepared (Sambrook et al. 1989); xylene loading buffer for DNA of low molecular weight and bromophenol blue for DNA of high molecular weight, thus avoiding band obscuration.

2.7.2 Molecular weight markers

Two molecular weight markers were used: (i) lamda phage *HindIII/EcoRI* digest (Promega) designated as ' λ ' in figures, and (ii) a 100 base pair ladder (Pharmacia Biotech, Uppsala, Sweden) designated as 'MW' in figures. As well as molecular weight sizing of bands, λ was also used to approximate DNA quantity.

2.7.3 Gel documentation

Ethidium bromide stained DNA bands were visualized using ultraviolet (UV) light. The results were recorded using gel documentation apparatus (Chromato-Vue, UV Transilluminator TM-20, UVP Inc., San Gabriel, CA, USA and Mitsubishi Video Copy Processor P65B, Tokyo, Japan) and relevant results were subsequently scanned using Adobe Photoshop 3.0 software (Macintosh) and a UMAX UC840 Max Vision Scanner at a resolution of 400 dots per inch. Image reproduction was enabled by pasting encapsulated postscript files into a desktop publishing file (PageMaker 6.5, Macintosh) for final

production of figures. Black and white print film (Polaroid, type 57) was also used for some agarose gels.

2.8 DNA PURIFICATION

2.8.1 Plasmid preparations

Small scale preparations of plasmid from 5 ml overnight LB cultures were prepared using the Wizard Minipreps DNA Purification System (Promega). Larger scale plasmid preparations used either Wizard Midiprep or Maxiprep (Promega) or Plasmid Maxi Kit (Qiagen, Hilden, Germany). In order to increase purity, as required for vaccinia homologous recombination, an additional phenol/chloroform extraction and ethanol precipitation (Sambrook et al. 1989) step was included. In later experiments, a desalting step was also added using membrane filtration (Microcon 50 or 100, Amicon) - see Figure 4.8.

2.8.2 Insert and vector purification prior to ligation

Inserts and vectors were purified using a variety of techniques.

- 1) Resin purification with the Wizard PCR Preps DNA Purification System (Promega) (for pSC11-ESAT-6 only).
- 2) Gel purification as described elsewhere (Sambrook et al. 1989). Briefly, 1% w/v agarose gel electrophoresis was performed and the appropriate band excised. Extraction from the agarose was achieved using either: (i) low melting point agarose (for pSC11-ESAT-6 cloning); or (ii) electroelution (all other cloning). This latter technique utilised $\frac{3}{4}$ " dialysis tubing (Sigma) containing the gel fragment and approximately 500 μ l of 1% w/v TAE; the tubing was sealed by two freezer bag clips. This was then placed into the buffer in the gel tank and eluted from the agarose. Micropure spin columns (Amicon) were also tried but this technique was abandoned (see Figure 4.8b and Section 4.2.3.2 for details).
- 3) Phenol chloroform extraction and ethanol precipitation (Sambrook et al. 1989).
- 4) Sephadex G-50 Fine columns (Pharmacia Biotech) were used for removal of the intervening oligonucleotide fragment following double digest of M13 bacteriophage cloning (Sambrook et al. 1989).
- 5) Spin column membrane filtration using Microcon 50 or 100 (Amicon) were used for: (i) enzyme removal (Micropure EZ insert); (ii) buffer exchange; (iii) DNA concentration, and (iv) removal of oligonucleotide fragment following double digest of vector (Figure 4.8a).

2.8.3 Nucleic acid quantification by spectroscopy

Spectroscopy was performed using a GeneQuant II spectrophotometer, Pharmacia Biotech, LKB Biochrom, Cambridge, UK for DNA and RNA samples where an assessment of purity and a more accurate estimate of quantity was required (first strand cDNA synthesis and plasmid preparation prior to transfection, *in vitro* transcription/translation and sequencing). Optical densities at 260 and 280 nm (OD_{260} and OD_{280} respectively) and the ratio OD_{260}/OD_{280} were recorded for a given dilution of sample. The quantity was calculated and purity assessed as described elsewhere (Sambrook et al. 1989).

2.9 PCR

2.9.1 Equipment and reagents

Thermal cycling was performed using a PHC-3 Techne thermal cycler. Working solutions of individual dNTPs (10 mM) and mixed dNTPs (2 mM) were stored at -20°C . The PCR mix consisted of: dNTPs (final concentration of 0.2 mM each); primers (final concentration of 0.2 mM each); template (usually 250 ng for cloning purposes and variable for diagnostic purposes); DNA polymerase (2.5 U of *Pfu* or *Taq*) plus $\times 10$ reaction buffer; plus 2 mM magnesium (for *Taq* only); and nanopure water up to a final volume of 100 μl for cloning purposes and 20–50 μl for diagnostic purposes. Reactions were performed using thin-walled 250 μl microfuge tubes.

2.9.2 Hot start PCR

A 'hot start' was used in most reactions by pre-sealing the primer pair under a wax pellet. This was achieved by adding the primer pair to the PCR tubes followed by the pellet. The tubes were then heated to 80°C for 5m then placed on ice. Initially, stock tubes containing pre-mixed primers under wax were stored at -20°C . Later, all reagents including primers were freshly made up and kept on ice to improve PCR yield.

2.9.3 Amplification of *esat-6* for cloning into pSC11

Plasmid pAA249, containing *esat-6*, was used as the template. For *Pfu* Polymerase, the cycle conditions were: initial temperature 95°C 5m, 40°C 5m, 74°C 30s; then 25 cycles of 95°C 20s, 45°C 1m, 74°C 30s; and extension of 75°C 5m. For *Taq* Polymerase, cycle conditions were similar except the annealing temperature was 45°C for the first cycle and 50°C for subsequent cycles and the extension temperature was 72°C throughout.

2.9.4 Amplification of genes encoding the Ag85 complex and ESAT-6

PCR amplification of cloning inserts was enabled using the appropriate plasmid template (Section 2.4.2) and primer pair (Table 2.1 and Section 2.5.3.2). Where problems with amplification were encountered, a variety of conditions were assessed in order to optimise both quantity and sequence fidelity. These included:

- (i) varying template and/or primer quantity;
- (ii) hot versus cold start;
- (iii) increasing melting temperature from 95 to 98 °C in order to reduce potential secondary structural problems;
- (iv) addition of 2% v/v glycerol or 10% w/v dimethylsulphoxide (DMSO) (Hybri-Max, Sigma) also in order to reduce potential secondary structural problems;
- (v) varying annealing temperature (range assessed 45-55 °C); and
- (vi) a switch from *Pfu* to *Taq* polymerase.

Following optimisation, reactions using *Pfu* polymerase were performed using the PCR mix as described in Section 2.9.1, a hot start and the following cycle protocol: melting temperature 95 °C 5m; 25 cycles of 95 °C 30s, annealing 50 °C 1m (except 55 °C for primer pairs 05/06, 07/08 and 11/12), extension 75 °C 30s followed by 75 °C for 5m. Reactions using *Taq* polymerase (primer pairs 01/02 and 03/04) were as described for *Pfu* with an annealing temperature of 50 °C and an extension temperature of 72 °C.

2.9.5 Making a digoxigenin-labelled *esat-6* probe

esat-6 was amplified using the same protocol described in Section 2.9.3 using *Taq* Polymerase. However, in this case 25% of the dTTP was replaced by digoxigenin-labelled-11-dUTP(Boehringer-Mannheim). The PCR product was run on a 1% w/v LMP agarose gel, the corresponding band cut was out and stored in 100 µl aliquots at -20 °C. In order to demonstrate successful digoxigenin-labelled probe, 10 µl aliquots of neat and three 10 fold dilutions of probe were blotted onto nylon membrane (Hybond-N, Life Science), baked for 2h at 80 °C and then subjected to a chemiluminescent protocol (described in Section 2.16.1.4). Successful digoxigenin-labelling of the probe is shown in Chapter 3, Figure 3.7.

2.10 T:A CLONING

The coding region, *esat-6* was inserted into pSC11 using T:A cloning as described by Marchuk and colleagues (Marchuk D et al. 1991). Figure 2.5 summarises the technique which takes advantage of the 3' terminal transferase activity of *Taq* Polymerase as over 90% of PCR product strands have a single 3' protruding dATP (A-tail). This overhang can be ligated to a cut vector with a complementary 3' dTTP overhang (T-tail). Ligation is more efficient than blunt-ended ligation and the risks of vector religation and insert concatamerisation are reduced.

2.10.1 T-tailing of the *Sma*I cut pSC11

pSC11 was *Sma*I digested, gel and resin-purified and then incubated with *Taq* polymerase, x10 reaction buffer, 4 mM Mg and 6 mM dTTP at 72 °C for 2h to produce T-pSC11. This was followed by further resin purification and estimation of DNA concentration using gel electrophoresis.

2.10.2 A-tailing of *esat-6*

The *Pfu* polymerase product was A-tailed as described for T-tailing but dTTP was substituted with dATP, thus producing A-*esat-6*. Amplification of *esat-6* using *Taq* polymerase resulted in an A-tailed product, which was used directly in the ligation reaction following purification.

2.10.3 Ligation of A-*esat-6* and T-pSC11, transformation and plasmid identification

Ligations were performed using T4 ligase, the appropriate reaction buffer and the addition of 1 µl of 1 mM ATP (stored in small aliquots at -70 °C) (Sigma) in a 10 µl final volume at 15 °C overnight. In the final experiment, the ligation mix was cut with *Sma*I, in order to reduce the background from vector religation. Following transformation (Section 2.3.1) and plasmid purification, pSC11-ESAT-6 could be identified using *Eco*RI digestion to identify recombinants and *Bsm*36I (or double digestion with *Eco*RI/*Pst*I) to identify orientation (see restriction map Figure 2.1).

2.11 DIRECTIONAL CLONING

Following T:A cloning of pSC11-ESAT-6, all subsequent cloning was directional, using non-complementary restriction sites at each end, thus permitting correct insert orientation and reducing the background vector religation.

2.11.1 Construction of p1108-tPA and pWR510-tPA

In half the constructs, coding sequences were placed downstream from a 90 bp region encoding the 21 amino acid signal sequence of tPA (Ny et al. 1984) and a multiple cloning site. This would permit expression of a target foreign protein with the tPA signal sequence leader (see Figure 2.2b). Two additional insertion plasmids, p1108-tPA (Figure 2.2c) and pWR510-tPA (Figure 2.3c) were therefore constructed.

2.11.1.1 Designing the tPA insert

In order to utilize the same oligonucleotide pair, the sense and antisense strands were designed to include: (i) the native start codon (and maintaining the same oligopyrimidine tract length to the ATG for pWR510 (Wang and Mullins, 1995)); (ii) a blunt 5' end suitable for cloning at pWR510 *Swa*I site and p1108 Klenow-filled *Bam*HI site; (iii) a multiple cloning site; and (iv) an *Eco*RI 4 bp 5' overhang for directional cloning and facilitating in frame fusion for the pWR510 His6 tag. The sense strand is shown in Figure 2.2b. The antisense strand included an additional GAAT overhang at the 5' end creating the 'cut' *Eco*RI sticky tail.

2.11.1.2 Cloning the tPA insert

- 1) The oligonucleotide pair consisted of an 86mer sense strand (tPA-OLI-1) and a 90mer antisense strand (tPA-OLI-2). These freeze dried preparations were dissolved in nanopure water to a concentration of 500 ng/ml.
- 2) 20 μ l of each were mixed in a microfuge tube, heated to 80°C in a water bath and allowed to cool slowly.
- 3) The hybridised product was gel and phenol/chloroform purified prior to cloning.
- 4) pWR510 was prepared by *Swa*I (3h at 25°C) followed by *Eco*RI digestion (2h at 37°C) using buffer H (Boehringer Mannheim) (see map, Figure 2.3).
- 5) p1108 was prepared by: (i) *Bam*HI digestion, (ii) Micropure EZ/Microcon step, (iii) Klenow fill (reaction consisting of: *Bam*HI cut vector, 2.5 μ l reaction buffer, 1 μ l

- (~5-10 units) Klenow enzyme, 1 μ l dNTPs (2 mM stock) in final volume of 25 μ l), (iv) phenol/chloroform step; (v) *Eco*RI digestion (see map, Figure 2.2); and (vi) Micropure EZ/Microcon step spun to a final volume of 10 μ l.
- 6) The ligation, transformation and plasmid preparation steps were performed using the optimised protocol (Section 2.11.2.3).
 - 7) In a repeat p1108-tPA ligation experiment, after the ligase reaction, the mix was cut with *Hind*III to reduce the religation background (this restriction site was present in vector but absent in recombinant).

2.11.2 Generation of PCR clones

2.11.2.1 Preparation of PCR-generated inserts prior to cloning

Between 300-500 μ l of amplified PCR product (3-5 reaction tubes pooled) was prepared as follows: (i) gel purified using electroelution; (ii) phenol/chloroform extracted and ethanol precipitated; (iii) double digested using appropriate restriction enzymes and universal buffer; (iv) enzymes were either heat inactivated, phenol/chloroform extraction step or removed with Micropure EZ (Amicon); and (v) membrane filtered using Microcon 50 or 100 (Figure 4.8a).

2.11.2.2 Preparation of vector prior to cloning

Approximately 1 μ g of plasmid vector was manipulated as described for steps (iii) to (v) for insert preparation (Section 2.11.1).

2.11.2.3 Development and identification of recombinants - the optimised protocol

The ligation, transformation and plasmid purification steps were carried out as described above (Sections 2.10.3, 2.3.2 and 2.8.1 respectively). However, at this stage, the protocol was standardised to include: (i) an insert to vector ligation ratio of 5:1 (weight ratio); (ii) adding 1-2 μ l of ligation mix containing 10 ng of vector DNA to SCS1 supercompetent cells; and (iii) plating out 200 μ l of transformed cells both neat and diluted 1 in 10. Successful recombinants were identified as described below.

- 1) Initially, screening was performed using PCR. The primer pair which had generated the insert was used with whole bacteria as the template and the *Taq* PCR protocol as described in Section 2.9.4, using 30 μ l reaction volumes. The bacteria were

picked from a discrete colony using a sterile toothpick. PCR positive clones were then subjected to plasmid purification and double enzyme digestion.

- 2) When cloning efficiency was reaching >80% (recombinants versus religations), the PCR step was omitted and 3-4 colonies were picked per ligation. Plasmid recombination was verified using double enzyme digestion.

2.11.3 Subcloning

Two subcloning experiments were performed. These included the transfer of: (i) *esat-6* from pSC11-ESAT-6 to M13 mp18 and mp19; and (ii) *85a* and *esat-6* from the p1108-tPA constructs to pWR510 and pWR510-tPA.

2.11.3.1 Preparation of plasmid-derived insert prior to cloning

Inserts were moved from recombinant plasmid to the target vector using the following steps: (i) approximately 2-5 µg of plasmid were double enzyme digested to cut out the relevant insert; (ii) the insert was gel purified and electroeluted; and (iii) further purification was performed using either phenol/chloroform extraction (M13 cloning) or Micropure EZ/Microcon membrane filtration (pWR510 cloning).

2.11.3.2 Construction of M13-ESAT-6

An *EcoRI/BamHI* fragment containing the *esat-6* gene was cut from pSC11-ESAT-6 and cloned into M13 mp18 and mp19. In order to enhance the efficiency of recombination, a Sephadex G-50 Fine column was used to remove the sticky-ended 20mer from the cloning site of the double-digested vector. The *EcoRI/BamHI* cut vectors and cut insert (see 2.13.1) were cloned using standard techniques. White plaques were picked and transferred to LB/tetracycline (50 µg/ml), recombinant phage grown overnight in XL-1 Blue *E. coli* and single stranded DNA prepared (Sambrook et al. 1989). Despite gel purification, there was some risk of contamination with a similarly sized fragment, the p7.5 promoter region (400 bp). Therefore, DNA preparations were subjected to PCR using the *esat-6* primers and the clones with strong PCR signals were selected for sequencing.

2.11.3.3 Construction of pWR510 and pWR510-tPA clones expressing 85A and ESAT-6

pWR510-85A, -tPA-85A, -ESAT-6 and tPA-ESAT-6 were generated using inserts (Section 2.13.1), cut vector (Section 2.12.2) and ligation/transformation (Section 2.12.3). Five transformants per ligation were screened using *NheI/EcoRI* digestion.

2.12 SEQUENCING

2.12.1 Manual sequencing of pSC11-ESAT-6 and M13 mp19-ESAT-6

2.12.1.1 Preparation of single stranded sequence

Two strategies for denaturing the double stranded pSC11 plasmid were utilised. (1) Plasmid DNA (3-5 µg in 18 µl volume) was added to 2 µl of 2M NaOH/50 mM EDTA and left at room temperature for 20m. 8 µl of 5M sodium acetate, pH 5.2 were added and this volume was ethanol precipitated. (2) Plasmid DNA (3-5 µg in 8 µl volume) was added to 16 µl of water, 8 µl of 2M NaOH, and 8 µl of 10 mM EDTA. The mix was then heated to 65°C for 5m. A pre-cooled mix of 12 µl of sodium acetate, 28 µl of water and 240 µl of ethanol was prepared, added to the denatured template and immediately transferred to dry ice in order to reduce renaturation. The mix was ethanol precipitated and resuspended in 14.5 µl of water providing enough for two sequencing reactions just prior to the annealing reaction. In the case of the M13 constructs, DNA was prepared according to a standard protocol (Sambrook et al. 1989).

2.12.1.2 Variations in the sequencing protocol

The Sequenase Version 2.0 DNA Sequencing Kit (USB/ Life Sciences) was used for sequencing. The protocols for annealing, labelling, and termination reactions were as described by the manufacturer. Variations in reactions included: (i) two labelling mix dilutions (1 in 5 and 1 in 15); and (ii) parallel use of the dITP labelling mix (1 in 5 dilution) to resolve compressions. In order to read close to the primer, manganese buffer was added and the labelling period shortened from 5 to 2m.

2.12.2 Cycle sequencing of p1108 and pWR510 constructs

PCR clones containing a mycobacterial gene were subjected to cycle sequencing using ABI PRISM Dye Terminator Cycle Sequencing (Perkin Elmer). The sequencing primers are listed in Table 2.2. Plasmid templates were purified, desalted and concentrated using Microcon membrane filtration (Amicon). Before an automated sequencer was available at

the London School of Hygiene & Tropical Medicine (LSHTM), an initial 15 reactions were performed at the School of Biological Sciences, University of Surrey, Guildford, UK. All subsequent reactions were performed at LSHTM. The manufacturer's protocol was followed, with the exception of two modifications based on 'in house' experience. These changes included: (i) a 50% reduction of terminator ready reaction mix (4 μ l used), 200 ng plasmid, 1.6 pmol of primer and water to a final reaction volume of 10 μ l; and (ii) a cycle protocol of 94°C 30s, 50°C 30s, 60°C 4m for 25 cycles. The 50°C annealing temperature was altered for some reactions, with the aim of setting this figure at approximately 5°C below the primer melting temperature.

2.13 CELL CULTURE

2.13.1 Plastics

Most tissue culture plastics were obtained from Greiner. These included: standardised culture flasks (T25, T75 and T175 representing 25, 75 and 175 cm² respectively), 60 x 15 mm individual Petri dishes and 6 well plates. The 24 and 96 well plates were obtained from Falcon, Becton Dickinson, Franklin Lakes, US. Nunc Lab-Tek Tissue Culture Chamber Slides (glass) were obtained from Life Technologies.

2.13.2 Cell culture media, serum and other additives

2.13.2.1 Media

All media were purchased from Life Technologies. Several types of medium were used in accordance with the cell culture requirements: (i) RPMI 1640 for human peripheral blood mononuclear cells (PBMC) and primary macrophage culture; and (ii) Dulbecco's Modified Eagle's Medium (DMEM), DMEM plus Glutamax I and Optimem I for TK-143 cell culture. Hanks Balanced Salt Solution (HBSS) was used for cell washes and as a diluent for virus.

2.13.2.2 Serum/plasma

Serum and plasma were sterile-filtered and stored at -20°C. Concentrations used in cell culture consisted of either: (i) 10% v/v autologous or human AB+ plasma for PBMC/macrophage culture; or (ii) 5% v/v heat-inactivated fetal calf serum (FCS, Sigma) for TK-143 culture.

2.13.2.3 Antibiotics and L-glutamine

Antibiotics were added unless otherwise specified: 0.5% v/v ampicillin (stock concentration of 10 mg/ml, Sigma) for PBMC/macrophage culture and 1% v/v penicillin/streptomycin mix (p/s) (stock penicillin concentration 1000 IU/ml, streptomycin concentration 1000 µg/ml, Life Technologies) for TK⁻ 143 culture. L-glutamine 1% v/v (L-glu) (Life Technologies) was added to RPMI and DMEM. This was not required for DMEM plus Glutamax I.

2.13.2.4 Complete media

For most cell culture work, complete medium for: (i) PBMC/macrophage culture included RPMI, ampicillin, L-glu and 10% v/v autologous plasma; and (ii) TK⁻ 143 cell culture included DMEM (or DMEM glutamax II), L-glu (DMEM only), p/s and 5% v/v FCS.

2.13.2.5 Additional reagents

5-Bromo-deoxyuridine (BUdR) was obtained from Sigma. Stock was made up to 2.5 mg/ml in PBS, warmed for 2m until dissolved, filter sterilised and stored at 4°C wrapped in silver foil. X-gal (Life Technologies) was freshly made up on each occasion by dissolving the powder in DMSO in a glass bijoux to make a stock concentration of 50 mg/ml. Two percent agarose (Type VII: Low Gelling; Sigma) was made up in serum-free antibiotic-free RPMI medium (Life Technologies), autoclaved and stored at 4°C. Mycophenolic acid (MPA) (Sigma), xanthine (Sigma) and hypoxanthine (Sigma) were each dissolved in 0.1M NaOH at stock concentrations of 10 mg/ml. The solutions were filter sterilised, aliquoted and stored at -20°C.

2.13.3 TK⁻ 143 tissue culture

The TK⁻ 143 cell line (ATCC CRL 8303), a human osteosarcoma cell line with a functional deletion of the thymidine kinase locus, was used to both construct rVV and propagate virus. The cell line was obtained from two sources: firstly from Dr T. Blanchard, who had initially received it from Dr H. Stauss, ICRF and secondly, also through Dr Blanchard, from Prof G. Smith, Sir William Dunn School of Pathology, Oxford. Cells were stored in the gaseous phase of liquid nitrogen. When required, cells were thawed rapidly in a water bath, washed and seeded into complete medium. On occasions, rapidly dividing cells were frozen down in complete medium containing 20% v/v FCS and 9% w/v DMSO.

Cell culture was maintained in T75 culture flasks in a humidified incubator at 37°C in the presence of 5% carbon dioxide. Passage was performed when the monolayer reached near confluence with a 1 in 5 (range 4-8) division of cells. Sub-culture entailed: (i) discarding the medium; (ii) washing the monolayer once with phosphate buffered saline (PBS) (Sigma), (iii) addition of 2 ml of trypsin/EDTA (Sigma) and incubating at 37°C until the cells were just non-adherent; (iv) addition of 6-8 ml of complete medium to inactivate the trypsin; (v) washing the cells twice in a 14 ml tube at 1350 revolutions per minute (rpm) (410 g) for 10m using a Mistral 3000 bench centrifuge (MSE, Crawley, UK); (vi) resuspending the cells in 2.5 ml (range 2-4 ml) and seeding a 0.5 ml aliquot into a new T75 flask containing 11.5 ml of warmed complete medium.

2.13.4 Human PBMC and primary macrophage culture

Ethical permission was obtained in order to venesect up to 50 ml of blood from consenting laboratory donors.

2.13.4.1 Isolation of PBMC

- 1) Venous blood was collected into 50 ml sterile conical tubes containing preservative free heparin (20 units/ml of blood). This was processed within 1-2h.
- 2) Heparinised blood was diluted 1:1 with warm RPMI.
- 3) For each 20-25 ml of diluted blood, 12 ml of ficoll (Histopaque - 1077, Sigma), at room temperature, was placed in a 50 ml tube and overlaid with the diluted blood. The ficoll gradients were then centrifuged at 1350 rpm (410 g) for 30m at room temperature and no brake.
- 4) Following centrifugation, 10 ml of 50% v/v plasma (per 50 ml tube) were collected to be used for complete medium.
- 5) PBMC were collected from the interface using a plastic Pasteur pipette (Alpha laboratories, Eastleigh, UK). Approximately 4 ml from each ficoll gradient were transferred and pooled into a 50 ml tube. This was then topped up with warmed HBSS and washed three times, spinning at 1350 rpm (410 g) for 10m and full brake.
- 6) The final pellet was then carefully resuspended in 1 ml of sterile-filtered complete medium and the cells counted using a counting chamber and 0.4% trypan blue (Sigma). The cells were finally resuspended at 1×10^6 cells/ml in complete medium ready for use either in a cytotoxic T lymphocyte (CTL) assay or macrophage experiments.

2.13.4.2 Isolation of primary human macrophages

Seven day primary human macrophage cultures were used for: (i) target cells in CTL assays; (ii) immunofluorescence of recombinant protein following rVV infection; (iii) immunocytochemistry of antigen-coated phagocytosed beads; and (iv) Ziehl-Neelsen (ZN) staining of BCG-infected macrophages.

For experiments (ii)-(iv), 400 μ l of 1×10^6 /ml PBMC were plated out into 8 well tissue culture glass chamber slides (Life Technologies). Non-adherent cells were gently washed away the following day. The remaining adherent cells were cultured for a total of 7 days. Given that the macrophage population accounts for approximately 10% of PBMC, it was estimated that each well contained $\sim 4 \times 10^4$ macrophages. Culture for CTL target cells is described below.

2.14 VACCINIA PROPAGATION AND QUANTIFICATION

Permission for genetic manipulation of the vaccinia genome was obtained both from the Health Safety Executive, Ministry of Health (HSE) and the Genetic Manipulation Advisory Group (GMAG) of LSHTM.

2.14.1 Viral preparation - crude stock

Crude viral stock was prepared for biochemical analysis and storage. Both wild-type (wt) vaccinia (VV) (strain WR) and recombinant vaccinia (rVV) were propagated by infecting a near confluent TK⁻143 cell monolayer. Typically, infection of a T175 flask was performed on a near-confluent monolayer as described below (volumes scaled down appropriately for T75 and T25 flasks).

- 1) The media was discarded
- 2) The monolayer was washed with 6 ml of warmed PBS
- 3) The virus was added at a multiplicity of infection (moi) of 0.05 in 2 ml HBSS and incubated for 1-2h at 37°C.
- 4) The virus inoculum was discarded and replaced with 28 ml of complete medium and incubated for 48-72h until a cytopathic effect (CPE) was clearly developed.
- 5) The virally-infected cells were scraped into the medium, transferred to a centrifuge tube and pelleted at 1350 rpm (410 g) for 10m.
- 6) The cells were resuspended in 2 ml of cold HBSS containing 0.1% w/v bovine serum albumin (BSA) or 10 mM Tris-HCl pH 9.0.

- 7) Virus was released by three freeze/thaw cycles using liquid nitrogen and a water bath. This was followed by 30s of water bath sonication.
- 8) Released virus was stored in 5-10 aliquots at -70°C .

2.14.2 Viral preparation - sucrose cushion-purified stock

Sucrose cushion-purified virus was used for animal immunisation and human *in vitro* assays. Preparation involved infection of eight T175 monolayers per rVV as described in Section 2.14.1 steps 1) to 4). The remaining procedure was carried out on ice.

- 1) Cells were pelleted at 1350 rpm (410 g) for 10m and resuspended in 2 ml of cold 10 mM Tris-HCl pH 9.0.
- 2) This suspension was homogenised with 20 strokes of a tight fitting Dounce homogeniser.
- 3) The nuclei were pelleted by centrifugation at 750 g for 5m.
- 4) The supernatant was recovered and kept on ice.
- 5) The pellet was resuspended in 10 mM Tris-HCl pH 9.0 and steps 2) to 4) were repeated. The supernatants were pooled.
- 6) 8 ml of supernatant were layered on 7 ml of autoclaved 36% w/v sucrose dissolved in 10 mM Tris-HCl pH 9.0. These sucrose cushions were prepared in U-bottomed centrifuge tubes suitable for a SW40 Ti swing bucket rotor (Beckman, High Wycombe, UK).
- 7) Centrifugation was performed at 14,400 rpm (25000 g) for 80m at 4°C in a Beckman L8-80 Ultracentrifuge.
- 8) The pellet was resuspended in a small volume of 1 mM Tris-HCl pH 9.0 (1-2 ml) and pooled with other pellets from the same preparation.
- 9) Sonication for 30s was performed using a cup sonicator (Sonicator, Ultrasonic Processor XL, Heat Systems, Farmingdale, NY, USA).

2.14.3 Viral titration - calculation of pfu/ml

The number of pfu/ml of viral stock was measured by sampling from one cryotube.

- 1) Six well plates were seeded with 8×10^5 TK-143 cells/well in 2 ml complete medium and allowed to grow overnight.
- 2) Monolayers were washed once with PBS and infected with five tenfold dilutions of stock virus in 0.5 ml of HBSS containing 0.1% w/v BSA (normal range 10^{-2} - 10^{-6} , but higher dilutions were performed with high titre stock).

- 3) The virus inoculum was replaced with 2 ml of complete medium and incubated for 48h.
- 4) The monolayers were washed with PBS. This was followed by adding 0.5 ml 0.2% w/v crystal violet stain per well and left to stand for 2m. The residual stain was removed and the plates allowed to dry.
- 5) The number of plaques per well for a given dilution was counted and the pfu/ml of stock virus was determined (Figure 2.6, see figure legend for details).

2.15 CONSTRUCTION OF rVV

This study utilised two similar techniques to construct rVV, both requiring lipid cationic transfection and recombinant virus selection. The transfection step was achieved using one of two cationic lipid transfection reagents: (i) lipofectin (Life Technologies) for construction of rVV-ESAT-6 using the pSC11 construct; and (ii) DOTAP (Boehringer Mannheim) for all other rVV. The methodology for cell transfection and virus homologous recombination was derived from the manufacturer's instructions and from Mackett (1995). Recombinant selection for the pSC11-derived rVV utilised *lacZ* expression and negative selection due to the *tk* phenotype. p1108 and pWR510-derived constructs were positively selected using guanine phosphoribosyl transferase gene (*gpt*) expression.

2.15.1 Construction of pSC11-derived rVV-ESAT-6

2.15.1.1 Transfection using lipofectin

A recombination mix containing rVV-ESAT-6 was obtained using the following protocol.

- 1) A near confluent TK⁻ 143 cell monolayer in a T25 flask, seeded the previous night, was infected with wt VV at a moi of 0.1.
- 2) The virus inoculum was discarded and the monolayer washed twice prior to adding the lipid-DNA complex.

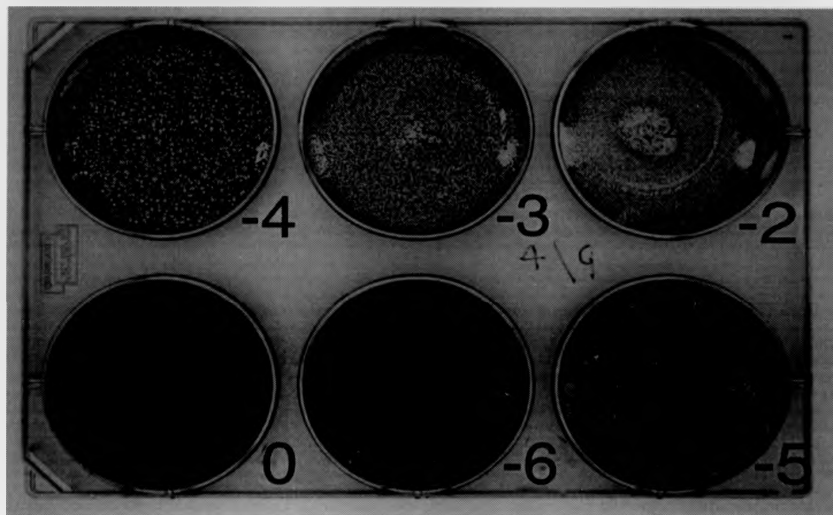


Figure 2.6 Vaccinia plaque assay

Measurement of viral titre was performed by seeding a 6 well plate with TK-143 cells the previous night and infecting 5 wells with a series of tenfold dilutions, according to the suspected titre of the virus. In this case, an initial dilution of 1 in 100 was made (the well infected with this concentration is labelled -2) with increasing dilutions to well -6. The remaining well (labelled 0) was mock infected with virus diluent alone. Plaques were counted after 48 hours of culture, using crystal violet to stain the monolayers.

- 3) In one tube, 1 µg of pSC11-ESAT-6 was added to serum-free and antibiotic-free medium, with a final volume of 260 µl. In a second tube, 26 µl of lipofectin reagent (Life Technologies) was made up to the same volume with the same diluent. The contents of the two tubes were mixed and left for 10m at room temperature.
- 4) Two millilitres of antibiotic-free, serum-free medium was added to the lipid-DNA complex. This was then added to the freshly washed T25 monolayer.
- 5) The flask was incubated for 5h and then topped up with 2 ml medium containing 5% v/v FCS.
- 6) The flask was incubated for a further 36h and virus harvested as described above.

As a positive control, the same experiment was repeated using pSC11-MOMP, containing a chlamydial gene insert (Blanchard et al. 1993).

2.15.1.2 Purification of rVV-ESAT (*tk* and *LacZ* selection)

Two rounds of plaque purification were performed in order to achieve clonality and to separate rVV from contaminating wt VV. This was achieved as described below.

- 1) Six Petri dishes (60 x 15 mm) per recombination mix were seeded and cells were grown overnight in the presence of BUdR (25 µg/ml).
- 2) Monolayers were infected with three tenfold dilutions (10^{-2} - 10^{-4}) of the recombination mix and this was performed in duplicate.
- 3) After 2h, a 4 ml agarose overlay containing 1% w/v type VII agarose (Sigma) 25 µg/ml BUdR and 2.5% v/v FCS in medium was added.
- 4) The overlay was cooled to room temperature under foil (approximately 10m) and then incubated at 37°C for 48h.
- 5) A second agarose overlay containing 200 µg/ml X-gal was added and this was incubated for a further 2-6h, until blue plaques were visible.
- 6) A blue plaque was picked using a plastic Pasteur pipette and transferred to 1 ml of medium for subsequent virus release as described in Section 2.14.1 step 7).
- 7) Half of the released virus derived from the plaque was used to infect a further monolayer (2nd plaque purification) and steps 3) to 6) were repeated.
- 8) rVV derived from the second plaque was then grown up in the presence of BUdR (typically one T25 flask followed by one T75 flask). Viral titration was then performed prior to making stock.

2.15.2 Construction of rVV derived from p1108 and pWR510

2.15.2.1 Transfection using DOTAP

The recombination mix for 18 rVV was prepared as described below.

- 1) T25 monolayers, 90% confluent, seeded the previous night, were infected with wt VV at a moi of 0.1.
- 2) DOTAP (22 μ l) was added to a 15 ml polystyrene tube for each of the rVV recombination experiments and a 25 μ l volume containing 4 μ g of the appropriate recombinant plasmid was added to the DOTAP and gently mixed. Tubes were then left for 15m at room temperature.
- 3) Three millilitres of DMEM containing 5% v/v FCS (no antibiotics) were added to each of the lipid-DNA mixes.
- 4) The virus inocula were discarded from the T25 flasks 1h post-infection (pi). Following this, the lipid-DNA/medium mix samples were added to the appropriately labelled infected-cell monolayers.
- 5) The following morning the medium was replaced with complete medium and transfected cells were incubated for a further 24h.
- 6) The virus was harvested as described in Section 2.14.1 steps 7) to 8).

2.15.2.2 Purification of rVV (gpt selection)

Purification of rVV was achieved as described below.

- 1) Six well plate cell culture monolayers were prepared.
- 2) The complete medium was changed to DMEM containing 2.5% v/v FCS, 250 μ g/ml xanthine, 15 μ g/ml hypoxanthine and 5 μ g/ml MPA (MPA selection medium) and the cells incubated overnight.
- 3) Monolayers were infected with three tenfold dilutions (10^{-2} - 10^{-4}) of the recombination mix and this was performed in duplicate.
- 4) The inocula were discarded after 2h and MPA selection medium was added. The monolayers were cultured for a further 48h (an agarose overlay was not used).
- 5) The medium was replaced with DMEM containing 0.01% w/v neutral red and incubated for 2h.
- 6) Two plaques were picked per recombinant using a plastic Pasteur pipette. Each was transferred to 1 ml of HBSS 1% w/v BSA for virus release as described in Section 2.14.1 step 7).

- 7) Half of the plaque-derived released virus was used to infect a further monolayer (2nd plaque purification) and steps 4) to 6) were repeated.
- 8) The rVV was then grown up in MPA selection medium in a similar manner to Section 2.15.1.2 step 8).

2.16 CONFIRMATION OF rVV

2.16.1 Southern blotting of rVV-ESAT-6 genomic DNA using the digoxigenin-labelled *esat-6* probe

2.16.1.1 Extraction of genomic DNA from virally-infected TK⁻143 cells

A near confluent T75 monolayer was infected with rVV-ESAT-6 at a moi of 10:1 and cells were harvested after 24h. The cell pellet was resuspended in 0.8 ml of TE buffer and digested with the addition of 20 μ l proteinase K (5 mg/ml stock stored at -20°C) (Sigma) and 40 μ l 10% w/v sodium dodecyl sulphate (SDS) (BDH Electran). This was mixed and digested overnight at 37°C. To prevent SDS precipitation at low temperature, 40 μ l of 5M NaCl were added prior to a phenol/chloroform extraction purification step. The genomic preparation was then dissolved in 50 ml of water.

2.16.1.2 *Eco*RI digestion of genomic rVV and plasmid DNA

A 20 μ l aliquot of purified genomic DNA (~1 μ g/ μ l) was added to 67 μ l of water, 3 μ l of *Eco*RI and 10 μ l of enzyme reaction buffer and incubated for 3h at 37°C. The digest was then ethanol precipitated and resuspended in 10 μ l. A rVV- β gal DNA preparation, kindly provided by Dr D Conway, was used as a negative control. *Eco*RI digests of plasmids pSC11-ESAT-6 (positive control) and pSC11 were also included.

2.16.1.3 Gel electrophoresis and capillary transfer to a nylon membrane

The digests were run on a 1% w/v agarose gel and the image recorded with a UV fluorescing ruler *in situ*. DNA was then transferred by capillary action onto N-Hybond nylon membrane (Life Sciences) following a standard protocol. The membrane was then baked for 2h at 80°C.

2.16.1.4 Chemiluminescent hybridisation

Hybridisation was performed using the digoxigenin-UTP labelled *esat-6* probe (Section 2.9.5), anti-digoxigenin alkaline phosphate conjugate and Lumigen PPD (Boehringer

Mannheim). The chemiluminescent protocol was followed as per manufacturer's instructions and the signal was detected using ECL Hyperfilm (Life Sciences).

2.16.2 RT-PCR for recombinant *esat-6* transcripts

2.16.2.1 Isolation of RNA

The following protocol was adapted from Gough (1988) and required two solutions: (i) Solution 1 (final concentration) = 10 mM Tris-HCl, pH 7.5, 0.15M NaCl, 1.5 mM MgCl₂, 0.65% w/v nonidet P-40 (NP-40, Sigma) (stored as x10 solution at 4°C); and (ii) Solution 2 = 7M urea, 1% w/v SDS, 0.36M NaCl, 10 mM EDTA, 10 mM Tris-HCl pH 7.5 (stored at room temperature).

- 1) A near confluent T25 monolayer was infected with rVV-ESAT-6 at a moi of 20:1 and cells were harvested after 24h as described above
- 2) mRNA was isolated by resuspending the pellet in 200 µl of ice cold Solution 1 and 1 ml of RNasin (Promega). This was vortexed vigorously and centrifuged at 6,500 rpm (3300 g) for 5m to pellet the nuclei.
- 3) The supernatant was added to 200 µl of Solution 2.
- 4) A standard phenol/chloroform extraction step was carried out and the ethanol-precipitated RNA was dissolved in 50 µl of DEPC-treated water and assessed using agarose electrophoresis and spectroscopy.

2.16.2.2 First strand cDNA synthesis and PCR of cDNA

Reverse transcription of mRNA was performed using the SuperScript Preamplification System (Life Technologies) in accordance with the manufacturer's instructions. In this case, oligo(dT) was used for first strand cDNA synthesis. This was followed by PCR of cDNA using the ESAT-6 cloning primers (Section 2.5.3.1) and the *Taq* PCR protocol (Section 2.9.3), thus amplifying a predicted 292 bp product.

2.16.2.3 Appropriate controls

Given that the mRNA preparation would be contaminated with viral genomic DNA (VV is a cytoplasmic virus), the test samples were treated with deoxyribonuclease I, amplification grade (DNase) (Life Technologies) prior to the RT step. The relevant controls included: (i) no RT step without DNase treatment (contaminating rVV genome); (ii) no RT step with DNase treatment (efficacy of DNase treatment); (iii) RT step included and no DNase

treatment (combined signal from genomic and cDNA template); (iv) a plasmid incorporating *esat-6* (positive control); and (v) cDNA from rVV lacking *esat-6* (negative control).

2.16.3 Immunoblotting

Both dot blots and Western blots were performed to detect mycobacterial proteins (and β -gal) derived from rVV and mycobacterial culture filtrate. Dot blotting was used to screen for efficacy of donated antibodies and permit their titration prior to Western blotting. It also provided a comparison between relative levels of expression of recombinant proteins. A chemiluminescent protocol, the ECL Western blotting Detection system (Life Sciences), was used for all immunoblotting.

2.16.3.1 Antibodies

Antibodies used in the detection of protein are listed in Table 2.3. Antibodies against mycobacterial proteins of the Ag85 complex and ESAT-6 were kindly donated by collaborating laboratories; the monoclonal antibody (mAb) against β -gal was purchased (see Table 2.3). Where data were unavailable, dot blots using different primary (1°) antibody dilutions were assessed in order to optimise antibody concentration for subsequent immunoblots (see example, Figure 2.7). Antibody dilution details for immunofluorescence and immunocytochemistry are also listed in Table 2.3.

2.16.3.2 Protease inhibitor mix

In order to prevent protein degradation, a mix of different protease inhibitors was used when harvesting virally-infected cell lysates. This included two x200 stock solutions, PI-A and PI-B. Individual reagents were made up, as described in Appendix A, Table A.3. PI-A (aqueous) was made by mixing 1 volume (vol.) each of EDTA, EGTA and NEM plus 2 vol. of water. PI-B (organic) was made by mixing 3 vol. of PMSF with 1 vol. each of Pepstatin and TPCK and warming in a water bath at 65°C until dissolved. These solutions were stored in 1 ml aliquots at -20°C.

2.16.3.3 rVV recombinant protein expression under p7.5 control

Protein extraction was performed using lysis buffer and the protease inhibitor mix (see Appendix A). The lysis buffer consisted of: 50 mM Tris-HCl (pH 8.0), 150 mM NaCl,

0.02% w/v sodium azide and 1% w/v NP-40. Five microlitres each of PI-A and PI-B were added to 990 μ l of lysis buffer just prior to use.

Antibody name	Protein recognised	Antibody type (origin)	Optimal dilution*	2° antibody (dilution)	Source of 1° antibody
HYB76-8	ESAT-6	mouse mAb (protein A purified)	1 in 1000	P260 [§] (1 in 1000)	P Andersen, Copenhagen, Denmark
TD-17	Ag85 complex	mouse mAb (supernatant)	1 in 20	P260 [§] (1 in 1000)	K Huygen, Brussels, Belgium
			immuno-fluorescence 1 in 2	anti-mouse FITC [#] (1 in 500)	
TD-32	Ag85 complex	mouse mAb (supernatant)	1 in 20	P260 [§] (1 in 1000)	K Huygen, Brussels, Belgium
Sera from Ag85 DNA vaccinated mice	Ag85 complex	4 mouse polyclonal sera 1) 85A, 2) 85B, 3) 85C & 4) 85A/B/C triple vaccinated (neat sera)	1 in 1000	P260 [§] (1 in 1000)	K Huygen, Brussels, Belgium
K92	Ag85 complex	rabbit polyclonal	1 in 1000	P217 [§] (1 in 1000)	M Harboe, Oslo, Norway
			immuno-cytochemistry 1 in 100	P217 [§] (1 in 100)	
K93	Ag85 complex	rabbit polyclonal	1 in 1000	P217 [§] (1 in 1000)	M Harboe, Oslo, Norway
Anti- β -gal	β -gal	mAb (Protein A purified)	1 in 5000	P260 [§] (1 in 1000)	Boehringer Mannheim

Table 2.3 Details of antibodies used

2°=secondary, P260=horseradish peroxidase conjugated to rabbit anti-mouse IgG, P217=horseradish peroxidase conjugated to goat anti-rabbit IgG, *=dilutions refer to immunoblotting unless otherwise stated, §=antibodies purchased from Dako, Glostrup, Denmark; #=gift of Dr P Kaye, LSHTM, London. FITC=fluorescein isothiocyanate.

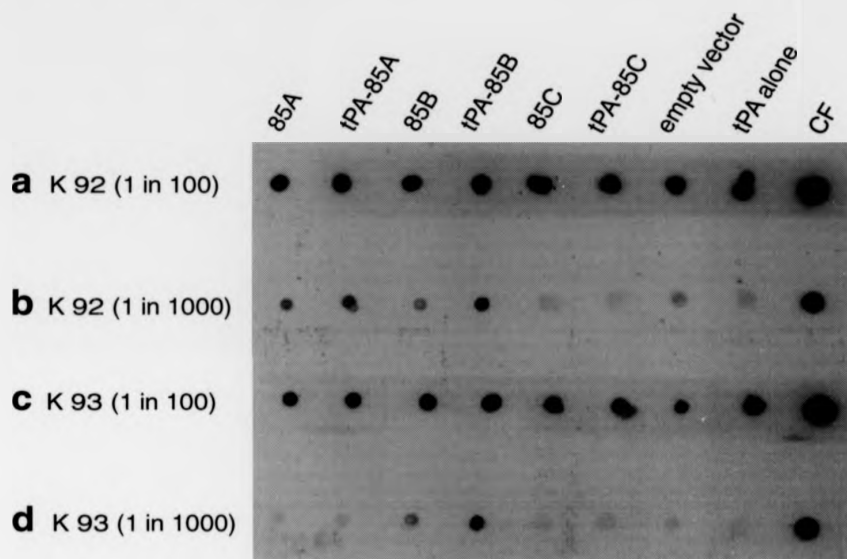


Figure 2.7 Assessment of two polyclonal antibodies raised against Ag85

Two rabbit polyclonal antibodies, K92 and K93 were assessed for their reactivity against rVV-Ag85 infected cell lysates and MTB CF (positive control). Undiluted 1 μ l aliquots were dotted onto nitrocellulose membranes and these were probed with the two antibodies using two different dilutions: **a**) & **c**) 1 in 100; and **b**) & **d**) 1 in 1000.

Near confluent T75 monolayers were infected with rVV at a moi of between 10:1 and 30:1. Mostly, these were incubated overnight. However, a time course pi (0, 1, 3, 5, 7, 18h) was also assessed for both rVV-ESAT-6 and rVV- β -gal. At harvesting, all reagents and the cell monolayer were kept on ice. Initially, the medium was discarded and the monolayer washed twice with PBS. The cells were then lysed using 550 μ l of lysis buffer plus protease inhibitors, scraped from the flask and transferred to a microfuge tube. This was then spun at 12000 rpm (11600 g) for 2m and the supernatant stored at -70°C . Subsequently, protein concentration was measured using the Bradford method (Maizels et al. 1991).

2.16.3.4 rVV recombinant protein expression under T7 control

Expression of the pWR510-derived rVV required co-infection with a rVV construct expressing T7 polymerase (vTF7-3, ATCC VR-2153). Thus, monolayers were co-infected with an equal titre (moi 10:1 each) of pWR510-derived rVV (foreign insert under the control of the T7 promoter; designated rVVpT7) and vTF7-3. Protein extraction was performed in a similar manner to 2.16.3.3

2.16.3.5 SDS protein gel electrophoresis (SDS-PAGE)

Protein gel electrophoresis was performed on a minigel system (Hoefer Mighty Small, Hoefer Pharmacia Biotech, San Francisco, USA) using standard SDS-PAGE (Sambrook et al. 1989). Polyacrylamide gels (12% w/v) were made using bis acrylamide:acrylamide (1:37.5) (Sigma). Gel preparation and electrophoresis solutions are described elsewhere (Sambrook et al. 1989). In some circumstances, a large gel system (Hoefer SE400) was employed to enhance resolution or to identify weak signals.

In most cases, 5-10 μ l of protein (rVV cell lysate, mycobacteria culture filtrate or *in vitro* transcription/translation product) were run under reducing conditions with the addition of 5% w/v 2-mercaptoethanol (Sigma) just prior to 5m boiling in SDS loading buffer. Size was recorded using a colour Low Range Molecular Weight (MW) marker (45 kDa, 29 kDa, 20 kDa, 14 kDa and 6.5 kDa) (Sigma). Where appropriate, replicate gels were run, one for Coomassie Blue (BDH) staining and the other, for immunoblotting.

2.16.3.6 Protein transfer and dot blotting

For SDS-PAGE, protein transfer to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) was performed using a semi-dry immunoblotter (the Semi-Phor, Hoefer Pharmacia Biotech) (Sambrook et al. 1989). A stack was made to permit electrophoretic transfer of proteins from the gel to 0.45 micron nitrocellulose membrane. The stack was composed of a Mylar mask, just smaller than the gel. This was placed on the anode and covered by three pieces of blotting paper soaked in transfer buffer. The blotting paper was then covered with a piece of nitrocellulose membrane, wetted with water (30s) then pre-soaked in transfer buffer (5m), followed by the gel and three further pieces of soaked blotting paper. The electricity supply was set at a current of 0.8 mA per cm² for 2h. Transfer was confirmed by visualising the colour MW marker bands on the nitrocellulose membrane. In addition, some experiments included the direct transfer of protein by dotting 1 µl volumes onto nitrocellulose membrane, usually with twofold protein dilutions. The membrane was left to dry (30m at room temperature) and then subjected to the same protocol as the Western immunoblot.

2.16.3.7 Signal detection

The ECL Western blotting Detection system (Life Sciences) was used in accordance with the manufacturer's instructions. Following initial optimisation three modifications were made. These included: (i) overnight incubation with 1° antibody in most cases (or occasionally for 2h at room temperature); (ii) use of 5% w/v milk powder (Marvel, Tesco's Supermarket) as a blocking agent; and (iii) inclusion of the blocking agent with both 1° and 2° antibody incubations. A series of steps were followed, using gentle rocking, as described below.

- 1) The nitrocellulose membrane was blocked for 2h using 5% w/v milk powder dissolved in PBS-T (PBS and 0.1% v/v Tween 20).
- 2) Five washes with PBS-T were performed (2 rinses followed by x3 10m washes).
- 3) 1° antibody diluted in blocking agent was incubated overnight at 4°C (or 2h at room temperature).
- 4) Five washes, as for step 2.
- 5) 2° antibody diluted in blocking agent was incubated for 1h at room temperature.
- 6) Five washes, as for step 2.
- 7) In the darkroom, the substrate reagents 1 and 2 were mixed (1 ml of each per minigel membrane) and then overlaid on the membranes for 1m. The membranes

were then briefly drip dried (10s) and quickly sandwiched between two clear plastic sheets (overhead projection sheets). Bubbles were carefully pushed to the edges of the membrane.

- 8) The membrane/plastic sheet sandwich was then placed in a film cassette and an initial exposure of 30s was performed using ECL Hyperfilm. Repeat exposures were taken according to the intensity of the signal. No increase in signal was noted for exposures greater than 30m.

2.16.3.8 Peptide N-glycosidase treatment of recombinant Ag85B

Deglycosylation of rVV-derived recombinant Ag85B was performed using peptide N-glycosidase (N-Glyconase, Genzyme, Cambridge, MA, USA). The protocol was based on the example described by manufacturer.

- 1) The sample (20 μ l) was boiled with 0.5% w/v SDS and 50 mM 2-mercaptoethanol in Tris-HCl pH 8.0
- 2) To a 10 μ l aliquot, the following reagents were added: 5 μ l of 7.5% w/v NP-40, 1.2 μ l (0.3 U) N-Glyconase and water to a final volume of 30 μ l.
- 3) The reaction mix was incubated overnight at 37°C.
- 4) The following day, the reaction was stopped by boiling for 5m. Samples were then run on SDS-PAGE or stored at -70°C.

2.16.4 Immunofluorescence

In order to confirm rVV expression in the target cells (primary cultured macrophages) used in the CTL assay, immunofluorescence was performed using an 'in house' protocol from the laboratory of Dr P. Kaye. Seven day cultured macrophages were prepared in an eight well tissue culture chamber slide as described in Section 2.13.4.2. The macrophages were infected with rVV-tPA-85A, rVV-85A and rVV-tPA alone at two moi (25:1 and 5:1) i.e. 6 wells plus 2 wells left uninfected. Eighteen hours pi the macrophages were then treated according to the following protocol.

- 1) The adherent cells were washed three times with PBS and very briefly rinsed with water.
- 2) Cells were fixed for 30m in freshly made-up 4% w/v paraformaldehyde in PBS pH 7.2.
- 3) Three washes were performed with PBS.

- 4) Quenching was performed with 50mM NH_4Cl in PBS for 10m.
- 5) The cells were rendered permeable with 0.01% w/v saponin in PBS 0.1% w/v BSA for 30m.
- 6) A further three washes were performed with PBS containing 0.01% w/v saponin.
- 7) Blocking was carried out using 50% v/v horse serum for 15m.
- 8) Step 6 was repeated.
- 9) The cells were then incubated with 1° antibody in PBS (Table 2.3) for 1h (1° antibody omitted from one of the uninfected wells).
- 10) Step 6 was repeated.
- 11) The cells were then incubated with 2° antibody in PBS (Table 2.3) for 45m.
- 12) Two washes were performed followed by rinsing with water.
- 13) The cells were covered with Vectashield mounting medium containing 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc., Burlingame, CA, US) and a glass coverslip.
- 14) Photographed was carried out using fluorescence microscopy (Zeiss Axioplan and MC100 camera, Zeiss, Oberkochen, Germany) with appropriate filters for both nuclear (DAPI) blue staining and recombinant protein (FITC-conjugated antibody) green staining.

2.17 T7 COUPLED TRANSCRIPTION/TRANSLATION

In vitro transcription and translation was performed in a single step using the TNT T7 Coupled Reticulocyte Lysate kit (Promega). The vaccinia insertion plasmid, pWR510, contained the appropriate elements for cell-free protein expression of *Ag85* genes and *esat-6* cloned downstream from the T7 promoter (Figure 2.3). The system was manipulated to compare products of the genes, both with and without the tPA coding sequence and also with and without the presence of microsomes.

2.17.1 Transcription/translation in the absence of microsomes

- 1) Eight pWR510 constructs were assessed (85A, 85B, 85C and ESAT-6 both with and without the tPA signal sequence). The reaction was carried out in accordance with the manufacturer's instructions, although the mix was scaled down by 50%.

TNT rabbit reticulocyte lysate	12.5 μl
TNT reaction buffer	1 μl
TNT T7 RNA polymerase	0.5 μl

amino acid mixture minus methionine, 1 mM	0.5 μ l
35 S-methionine (between 1-3 μ l depending on activity)	20 μ Ci
RNasin ribonuclease inhibitor, 40 u/ μ l	0.5 μ l
DNA template	<u>0.5 μg</u>
DEPC-treated water	final vol. 25 μ l

- 2) Following a 90m incubation at 30°C, samples were heated at 80°C for 5m to stop the reaction.
- 3) A 5-10 μ l sample from each reaction was subjected to 12% w/v SDS-PAGE. The remainder of the samples were stored at -70°C.
- 4) Following electrophoresis, the gel was dried and autoradiography performed with 4-48h exposures, depending on the strength of the signal.

2.17.2 Transcription/translation in the presence of microsomes

- 1) Six pWR510 constructs were assessed (85A, 85B, and ESAT-6 both with and without the tPA signal sequence). Ultracentrifugation of the products permitted comparison between membrane and non-membrane associated fractions. The reaction was carried out in accordance with the manufacturer's instructions in a manner similar to when there were no microsomes. The mix used in these experiments was as for 2.17.1, except 2.5 μ l of canine pancreatic microsomal membranes was included in the reaction mix.
- 2) Following a 90m incubation at 30°C, samples were cooled on ice.
- 3) Samples were then transferred to U-bottomed centrifuge tubes suitable for the Beckman TL 100.1 fixed angle rotor and spun at 50 000 rpm (88760 g) for 1h in a Beckman Optima TL Ultracentrifuge. The supernatant was recovered and the pellet resuspended in 25 μ l of RIPA buffer (Mackett, 1995). Samples were stored at -70°C until SDS-PAGE and autoradiography (2.17.1, step 4).

2.18 T CELL AND MACROPHAGE STUDIES

rVV expressing the mycobacterial secreted proteins were used to infect human macrophage target cells in a 6 hour CTL assay. Live BCG was used to obtain a restimulated CTL population (the effectors) (Turner and Dockrell, 1996). However, as this allows only restimulation of CTL recognising Ag85 epitopes, alternative strategies were considered. One of these strategies included the use of latex beads coated with mycobacterial culture

filtrate for macrophage phagocytosis and MHC class I antigen presentation. Preliminary CTL assays and a study with culture-filtrate coated latex beads were performed.

2.18.1 CTL assay

A ^{51}Cr release assay was performed as developed within our laboratory (Turner and Dockrell, 1996). The protocol included: (i) 7 days restimulation of PBMC CTL with live BCG; and (ii) 7 days culture of autologous macrophages with addition of antigen (BCG or rVV) the evening before the 6h release assay. The protocol is described below. An example of the target plate plan is also given (Figure 2.8).

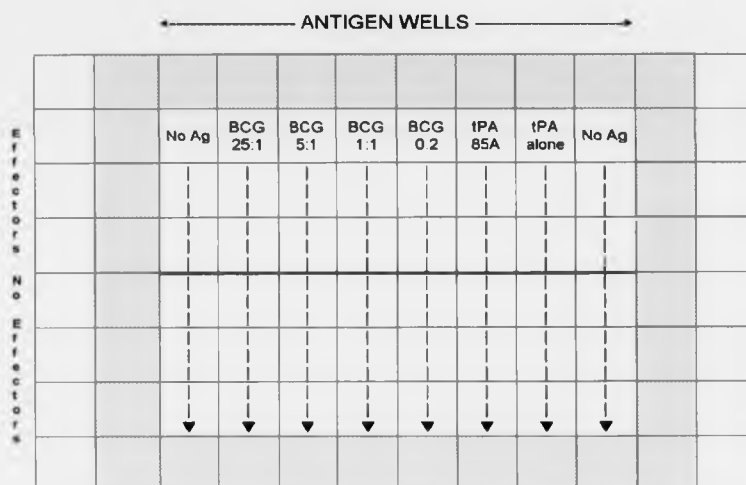


Figure 2.8 Example of plate plan

Shaded area = medium alone to prevent drying. tPA-85A and tPA alone=rVV infected target cells (moi 25:1).

day 0 Target and effector cells

- 1) PBMC were prepared at a dilution of 1×10^6 cells/ml as described in Section 2.13.4.1.
- 2) For use as target cells 150 μl aliquots of PBMC were pipetted into a 96 well U-bottomed plate in accordance with the plate plan. A 'hedge' of medium alone was placed around the PBMC wells to limit drying.

- 3) For use as effector cells 2 ml aliquots of PBMC were pipetted into 24 well plates and antigen (BCG) was added at a variety of moi (25:1, 5:1, 1:1 and 0.2:1). Subsequent experiments used a moi of 1:1.

day 1 Target cells

- 4) Non-adherent cells were removed by gentle pipetting up and down 5 times, removal of 100 μ l from each well and replacement with complete medium.

day 6 Target cells

- 5) A 100 μ l sample of medium was removed from each well and discarded.
- 6) Aliquots of 25 μ l of complete medium containing either rVV-tPA-85A, rVV-tPA alone (negative control), BCG (positive control), or complete medium alone (negative control) were added to each well according to the plate plan. Early studies used a variety of moi: (i) rVV - 25:1 and 5:1; and (ii) BCG - 25:1, 5:1, 1:1 and 0.2:1. Subsequent studies used BCG at 5:1.
- 7) An aliquot of 25 μ l of 2 μ Ci ^{51}Cr in complete medium was added to each well. The plate was then incubated overnight.

day 7

- 8) Effectors cells The effector cells were harvested, pelleted and resuspended in 1 ml for counting. Depending on the experiment, cell counts were adjusted to obtain a variety of effector:target ratios; typically 25:1, 5:1 and 1:1.
- 9) Target cells The target cells were washed 4 times very gently by discarding all the medium and replacing with warm RPMI. An aliquot of 50 μ l of complete medium was added to each experimental well followed by 100 μ l of either effectors or complete medium alone to measure spontaneous release from infected target cells. The plate was then incubated for 6h.
- 10) The plate was spun at low speed (1100 rpm, 270 g) in a Mistral 3000 centrifuge for 4m.
- 11) The entire supernatant from each well was transferred to an LP2 tube (Alpha Laboratories), taking great care not to disturb the pellet. This enabled both the supernatant and pellet count to be assessed. The pellets were then lysed with 150 μ l 5% w/v SDS for 2h and the contents were transferred to LP2 tubes. Each tube was sealed with wax and the activity of the supernatant and pellet measured on a gamma counter (1480 Wizard 3", Wallac).

- 12) The percentage isotope release (IR) and the cytotoxicity (C) was calculated as:

$$\text{IR} = \frac{\text{CPM supernatant}}{\text{CPM supernatant} + \text{CPM pellet}} \times 100$$

and:

$$C = \% \text{IR effector well} - \% \text{IR for target+Ag alone well}$$

where the target+Ag alone wells were manipulated in an identical way (using the same antigen) except for the omission of effector cells.

2.18.2 BCG infection of macrophages - assessing moi

2.18.2.1 Calculation of CFU per ml

- 1) BCG suspended in RPMI was prepared as described in Section 2.3.2.1.
- 2) An aliquot was thawed, briefly vortexed and spun for 15s at low speed (1000 rpm, 80g) to pellet any grossly clumped organisms.
- 3) The supernatant was obtained and water-bath sonicated for 10s.
- 4) Four tenfold dilutions (10^{-4} - 10^{-8}) of the supernatant were performed and plated in replicate. Plates were then incubated for 4-6 weeks.
- 5) Colonies were counted and the CFU/ml of stock calculated.
- 6) At a later date, steps 2 to 5 were repeated for another aliquot and the average taken.

2.18.2.2 Direct assessment of moi

- 1) A seven day macrophage culture, in an 8 well tissue culture chamber slide, was prepared as described in 2.13.4.2.
- 2) BCG was prepared as described in Section 2.18.2.1 steps 2 to 3.
- 3) Macrophages were infected with BCG at four moi (25:1, 5:1, 1:1 and 0.2:1) based on the estimated number of macrophages per well (4×10^4) and the calculated CFU/ml (Section 2.18.2.1). Two separate batches of BCG were assessed.
- 4) The following day, infected cells were washed three times with PBS and then briefly rinsed with water, to wash away precipitated salt which might have taken up stain non-specifically.
- 5) Cells were fixed for 15m with acetone and the washing step repeated.

- 6) A modified ZN stain was performed (cold staining) and the cells were counterstained with TB Brilliant Green K (Difco).
- 7) The percentage of infected macrophages for each moi was assessed. A photographic record was taken.

2.18.3 Mycobacterial culture filtrate coated beads

2.18.3.1 Covalent coating of carboxylated latex beads

The method had been previously established in this laboratory and had been adapted from the 'carbodiimide method' as recommended by Polysciences, Northampton, UK

- 1) Aliquots of 100 μ l of 10% w/v latex solution (carboxylated polystyrene microparticles, 0.9 μ m in size) (Sigma) were added to two microfuge tubes. A wash step was performed by mixing the beads with 800 μ l of carbonate buffer (pH 9.6), centrifuging for 5m at 12 000 rpm (11600 g), and discarding the supernatant (the beads float at this pH). The washing step was repeated twice.
- 2) Three washes were then performed with 0.02M phosphate buffer pH 4.5. The beads were resuspended in 500 μ l of this buffer.
- 3) A 500 μ l volume of carbodiimide (cyanamide, Sigma) was added slowly, the tube mixed by inversion and left at room temperature for 4h with gentle agitation.
- 4) Three washes were performed using 0.02M phosphate buffer pH 7.4 to remove unreacted carbodiimide.
- 5) The bead pellets were resuspended in 200 μ g of either MTB ST-CF (from SSI) or BSA. They were incubated at room temperature with gentle agitation overnight.
- 6) A 20 μ l volume of ethanolamine (Sigma) were added for 1h to block any unreacted sites.
- 7) Three washes were performed with 0.02M phosphate buffer pH 7.4 to remove unbound antigen and ethanolamine.
- 8) The beads were then incubated with 1 ml of 0.25% w/v BSA in 0.02M phosphate buffer pH 7.4.
- 9) Three washes were performed with 0.02M phosphate buffer pH 7.4.
- 10) The pellets were finally resuspended in 1 ml of 0.02M phosphate buffer pH 7.4 containing 8 mg/ml NaCl, 10 mg/ml BSA and 5% v/v glycerol and stored at 4°C for up to 1 month. This constituted a final concentration of 1% w/v beads.

2.18.3.2 Immunocytochemistry of culture filtrate-coated beads

In order to assess successful antigen coating (and have an indication as to an appropriate quantity of beads per macrophage), immunocytochemistry of beads phagocytosed by macrophages was performed.

- 1) A seven day macrophage culture on an 8 well tissue culture chamber slide was prepared as described in 2.13.4.2.
- 2) On day 6, 40 μ l, 4 μ l and 0.4 μ l of 1% w/v culture filtrate-coated beads were added each to a well containing 4×10^4 macrophages. This was repeated for BSA-coated beads. Two wells were left without beads.
- 3) On day 7, macrophages were fixed with acetone and washed as described in Section 2.18.2.2 steps 2 and 3.
- 4) The cells were then blocked with 1% w/v BSA PBS for 1h.
- 5) A 1h incubation with 1° antibody (K92 at 1 in 100 - see Table 2.3) in 1% w/v BSA PBS was performed. In one of the bead-free wells, no antibody was added.
- 6) Three washes with PBS were performed.
- 7) A 1h incubation with 2° antibody (P217 at 1 in 200) in 1% w/v BSA PBS was performed.
- 8) A further three washes with PBS were performed.
- 9) A freshly prepared solution of diaminobenzidine (Sigma) was added for 15m. The slide was then washed and mounted.

3 Construction and expression of rVV-ESAT-6

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3.1 INTRODUCTION

The gene encoding the mycobacterial protein, ESAT-6, represented an attractive first choice for rVV construction. This protein is a key target recognised by immune T cells in a mouse TB infection model (Andersen and Heron, 1993) and there is indirect evidence that the same is true in humans (Boesen et al. 1995), as discussed in Section 1.4.3.5. The gene is absent from known strains of BCG and most environmental mycobacteria (Mahairas et al. 1996), thus a potential rVV-ESAT-6 construct would represent a useful tool which would be specific for immune responses to the *M. tuberculosis* complex.

The gene was provided as part of a cloning vector, pAA249, through a collaboration with Dr P. Andersen, Statens Seruminstitut (SSI), along with a mAb for detection of recombinant ESAT-6 and ST-CF containing the native protein as a positive control in immunoblotting. In return, the construct was to be made available to SSI for mouse immunology studies including protective efficacy.

The cloning strategy was selected on the basis of the laboratory expertise available at the time. Several rVV constructs expressing chlamydia proteins had recently been constructed (Dr T Blanchard) using the vaccinia insertion plasmid, pSC11. At that time, plasmid recombinants had been generated using a T:A cloning strategy (Section 2.10) of PCR-generated foreign gene inserts. In addition, rVV were constructed using TK⁻ 143 cells, lipofectin transfection, and BUdR negative selection with the addition of colour selection to exclude *tk*-spontaneous mutants (Section 2.15.1).

The construction of pSC11-ESAT-6 was performed using much the same strategy as that used for the chlamydia constructs. Chapter 2 details the methods used in the construction of rVV-ESAT-6. The methodology included: (i) PCR generation of the insert (Section 2.5.3.1 and 2.9.3); (ii) T:A cloning into pSC11 (Section 2.10); (iii) homologous recombination of the insert and selectable marker into the *tk* locus of the vaccinia genome (Section 2.15.1); (iv) selection and purification of rVV; (v) identification of gene insertion and transcription; and (vi) identification of gene expression.

3.2 RESULTS

3.2.1 Construction of pSC11-ESAT-6

After initial attempts at cloning *Pfu* polymerase-generated *esat-6*, pSC11-ESAT-6 was finally constructed using *Taq* polymerase. The protocol steps are summarised in Figure 2.5, with the omission of A-tailing for the *Taq*-generated products.

3.2.1.1 PCR of *esat-6*

Strong single bands of predicted size (292 bp) were visible for all reaction conditions, whether using *Taq* or *Pfu* DNA polymerases. Figure 2.5a shows a preparatory gel prior to gel purification; in this case, all bands came from the same PCR reaction.

3.2.1.2 Yields of tailed insert and vector prior to ligation

Initially, yields of purified A-ESAT-6 and T-pSC11 prior to ligation were unacceptably low (<10 ng/ μ l). This yield was increased to 10-200 ng/ μ l by changing the methodology as follows. In the case of T-pSC11, the concentration and overall yield were improved by omitting the gel purification step and switching from resin purification (Promega) to a phenol/chloroform extraction step, with final resuspension of the pellet in a small volume of water (10 μ l). In the case of A-ESAT-6, the yield was improved by switching from *Pfu* to *Taq* amplification, thus dispensing with the additional A-tailing. Figure 2.5e shows faint bands of the tailed insert and vector used in the final construction of pSC11-ESAT-6.

3.2.1.3 Transformation efficiency

Ten transformations, designated T1-T10, were performed prior to obtaining the recombinant pSC11-ESAT-6. The initial transformation (T1) produced numerous colonies (>400 on each plate). Plasmid miniprep purification and restriction enzyme digestion showed that most of these were due to contamination with the PCR template plasmid (the first PCR product preparations were not gel-purified prior to ligation). Subsequent transformations (T2-T8) produced much lower transformation efficiencies with 0-24 colonies, although a plasmid control (pUC18) consistently produced >200 transformants per plate. After optimising the system as previously described, two further transformations, T9 and T10, resulted in high efficiencies (>400 colonies per plate). T-pSC11 was also added to the ligation mix without insert and a high religation background was noted. This background was greatly reduced by cutting the ligation mix with *Sma*I prior to transformation; this site is present in uncut vector (or religated untailed vector) but absent in recombinants.

3.2.1.4 Cloning attempts

One hundred and sixteen plasmid preparations from transformations T1-T9 resulted in pSC11 alone or the contaminating PCR template, pAA249. Gel purification of A-ESAT-6 resulted in a reduction but not elimination of pAA249. In one cloning experiment, 25% of transformants contained this plasmid, despite gel purification of the insert. In transformation T10 (which used the *Sma*I cut ligation mix), three recombinants were identified from 24 minipreps. This is demonstrated in Figure 3.1a which shows an *Eco*RI digest cutting a 700 bp band from putative pSC11-ESAT-6 recombinants (see also plasmid map, Figure 2.1). The recombination event was confirmed using *Bsu*36I, *Eco*RI/*Pst*I and *Sma*I digestion. The *Sma*I digest showed loss of the this site in the recombinants. The other digests indicated that the orientation was correct in clones 15 and 19 and incorrect in clone 18 (Figure 2.1b and Figure 3.1b).

3.2.2 Sequencing of pSC11-ESAT-6 and M13 subclones

3.2.2.1 Double stranded sequencing of pSC11-ESAT-6

Double stranded sequencing produced incomplete sequencing data covering only the first 225 bp of the 292 bp insert (see Figure 3.2a, reactions: 'PSC11F.SEQ', 'PCR_F.SEQ' and 'PCR_R.SEQ'). Four further attempts to complete the sequence, using a reverse external primer with viral *tk* homology (Section 2.12.1 and Table 2.4), were unsuccessful. The function of the sequencing primers had been previously confirmed using other pSC11 recombinants (Dr T Blanchard, personal communication).

3.2.2.2 Construction of M13 mp18- and mp19-ESAT-6

Further analysis was permitted by sub-cloning into two M13 single stranded sequencing vectors, mp18 and mp19. Directional sticky-ended cloning was achieved by *Eco*RI/*Bam*HI double digestion of pSC11-ESAT-6 with gel-purification of a band slightly greater than 292 bp. Ligation with the cut M13 vectors was performed, followed by transformation (XL-1Blue strain) and screening of white plaques (Section 2.11.3.2). There was the potential for ligating a contaminating ~400 bp insert containing the p7.5 promoter (see Figures 2.1 and 3.3a). Recombinant clones with *esat-6* were therefore identified using PCR. A plaque was picked for the PCR template and the original insert primers were used to generate products

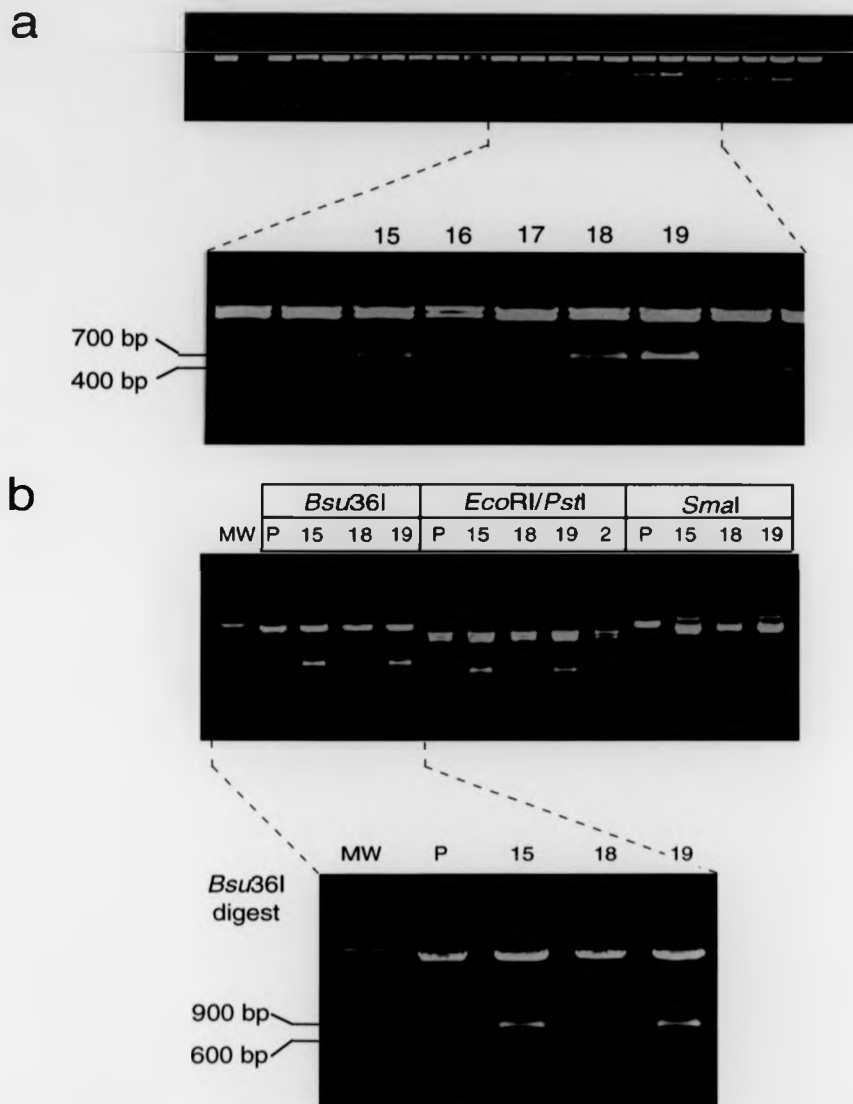


Figure 3.1 Identification of pSC11-ESAT-6 recombinants

a) Following ligation and transformation, 22 transformants were picked, plasmid 'minipreps' performed and the purified DNA digested with *Eco*RI and run on a gel. Three clones were consistent with recombination (15, 18 and 19), as suggested by the increase in fragment size from 400 to 700 bp. **b)** The 3 clones and a pSC11 control (P) were further digested with *Bsu*36I, *Eco*RI/*Pst*I, and *Sma*I. Successful recombination and orientation of insertion was verified. The detail of the *Bsu*36I digest shows a 900 bp band for clones 15 and 19 (correct orientation) and a 600 bp band for clone 18 (incorrect orientation). See restriction map in Figure 2.1 for details.

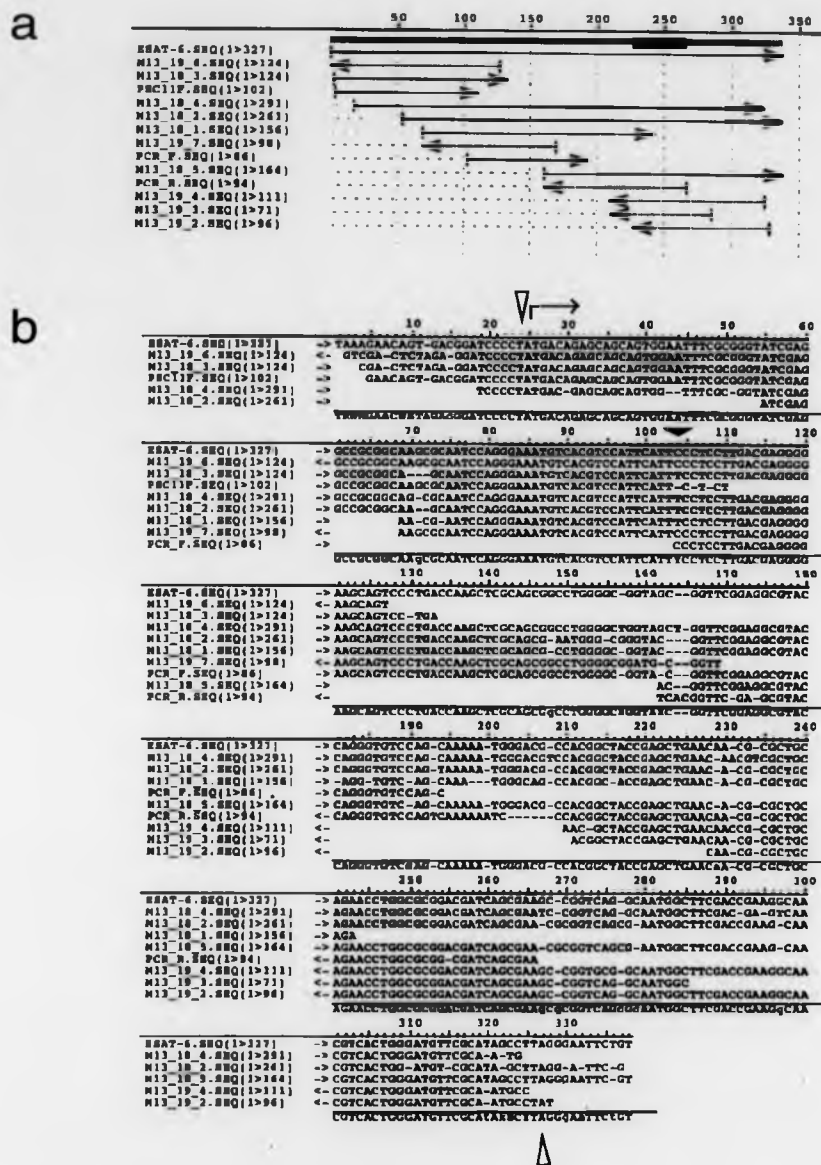


Figure 3.2 pSC11-ESAT-6 sequence data.

All sequences were entered into a computer alignment programme (Lasergene, Seqman) and the data was expressed both as: **a)** a strategy view, and **b)** an alignment view. The left hand list indicates names of the sequence runs and the arrow indicates the direction of the run. The first sequence, labelled 'ESAT-6.SEQ' represents the published sequence data (Sorensen et al. 1995). Other names refer to single-stranded runs (prefixed M13) or double stranded runs (all others). ∇ = T-tail, Δ = A-tail from AT cloning. \blacktriangleleft = C to T substitution in four runs from one M13_mpl8 clone.

of 292 bp in size (Section 2.5.4). Figure 3.3c shows that 6 out of 10 white plaques contained the insert for these M13 mp18 clones. M13 mp19-ESAT-6 clones were also generated (data not shown). Together, these recombinants permitted single stranded sequencing in both directions.

3.2.2.3 *Single stranded sequence data*

M13 mp18- and mp19-ESAT-6 sequencing was performed. Figure 3.2 shows the computer generated sequence strategy view and alignment. Figure 3.4 shows 4 examples of 13 runs. Of interest, an unequivocal substitution was present in one of M13 mp18 clones (C to T, position 104, Figure 3.2b). However, this was absent in sequence data from two other M13 clones (one mp18, the other mp19) and the double stranded sequencing, thus indicating a mutation in that M13 clone.

Several sequence conflicts in GC rich regions were identified following sequencing of M13 mp18-ESAT-6. These were all resolved by sequencing in the reverse direction using an M13 mp19-ESAT-6 clone. The sequencing data demonstrated successful T:A cloning at the *Sma* I site, with T and A residues flanking the amplified product at positions 24 and 327 respectively (Figure 3.2b).

3.2.3 Construction of rVV-ESAT-6

3.2.3.1 *Transfection and rVV purification*

The first attempt at transfection was successful for both rVV-ESAT-6 and the rVV-MOMP control (chlamydia gene encoding the major outer membrane protein) (Blanchard et al. 1993). Five plaques were picked from the rVV-ESAT plaque purification plates (two from the 10^{-2} plate and three from the 10^{-3} plate) and subjected to further plaque purification steps (Section 2.15.1.2). Following two rounds of plaque purification, one virus plaque was then used to infect a T25 monolayer which yielded ten aliquots of 1.5×10^6 pfu/ml. This was then used as a source for growing up stock virus.

3.2.3.2 *Achieving high pfu/ml stock*

In the early stages of this study, our laboratory had achieved relatively poor viral yields. In an attempt to optimise viral growth, probe sonication was compared with water bath sonication. A 2.7 fold increase in pfu was achieved using probe sonication (7×10^7 versus 2.6×10^7 pfu/T175 monolayer). However, the probe sonicator introduced the potential for

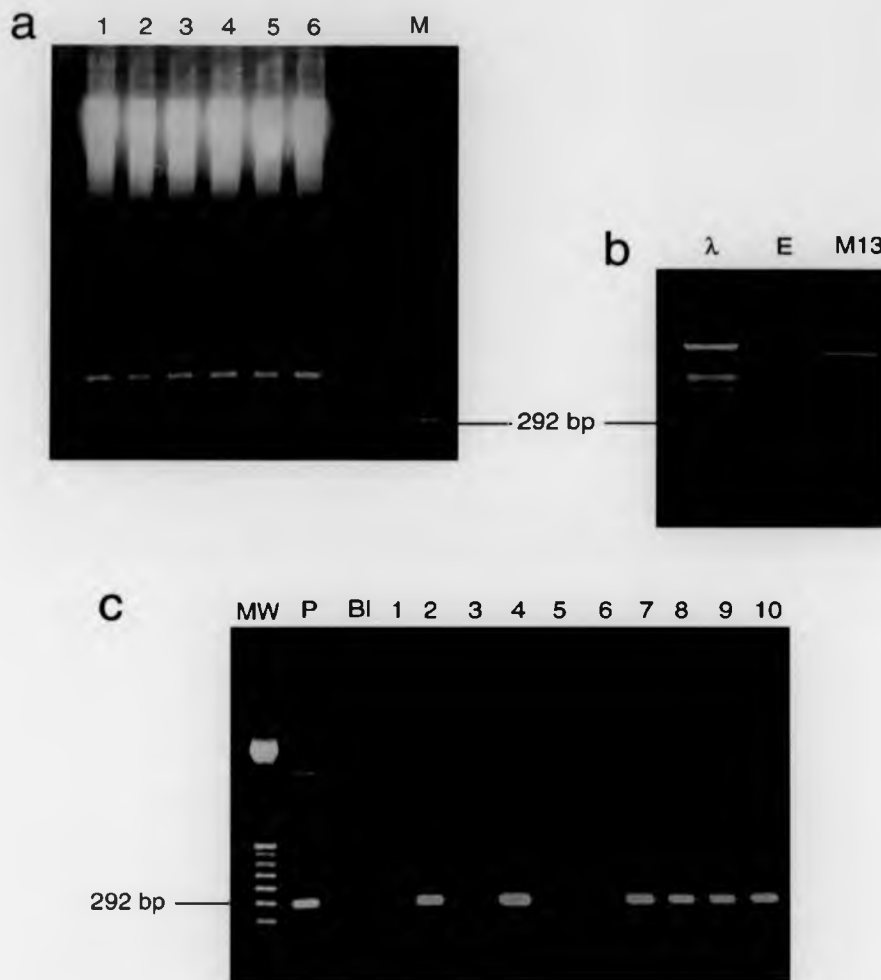


Figure 3.3 Cloning *esat-6* into the bacteriophage M13

a) pSC11-ESAT-6 was double digested with *EcoRI* and *BamHI* and the digest run on a preparatory gel (lanes 1-6). The lowest molecular weight bands (slightly larger than the 292 bp marker) corresponded to the fragment containing *esat-6*. These were cut from the gel, pooled and purified prior to cloning. **b)** The purified *EcoRI/BamHI* cut *esat-6* insert and the *EcoRI/BamHI* M13mp18 cut vector were subjected to agarose electrophoresis (lanes 'E' and 'M13' respectively). λ=lamba *EcoRI/HindIII* digest molecular weight marker. **c)** The ligation reaction was performed and 10 recombinant phage (white plaques) were picked for PCR verification of *esat-6* insertion and the PCR products electrophoresed (Lanes labelled 1-10). A blue plaque was included as a negative control (lane 'BI') and the *esat-6* cloning vector, pAA249, was used as a positive control (lane 'P'). MW=100 bp molecular weight marker.

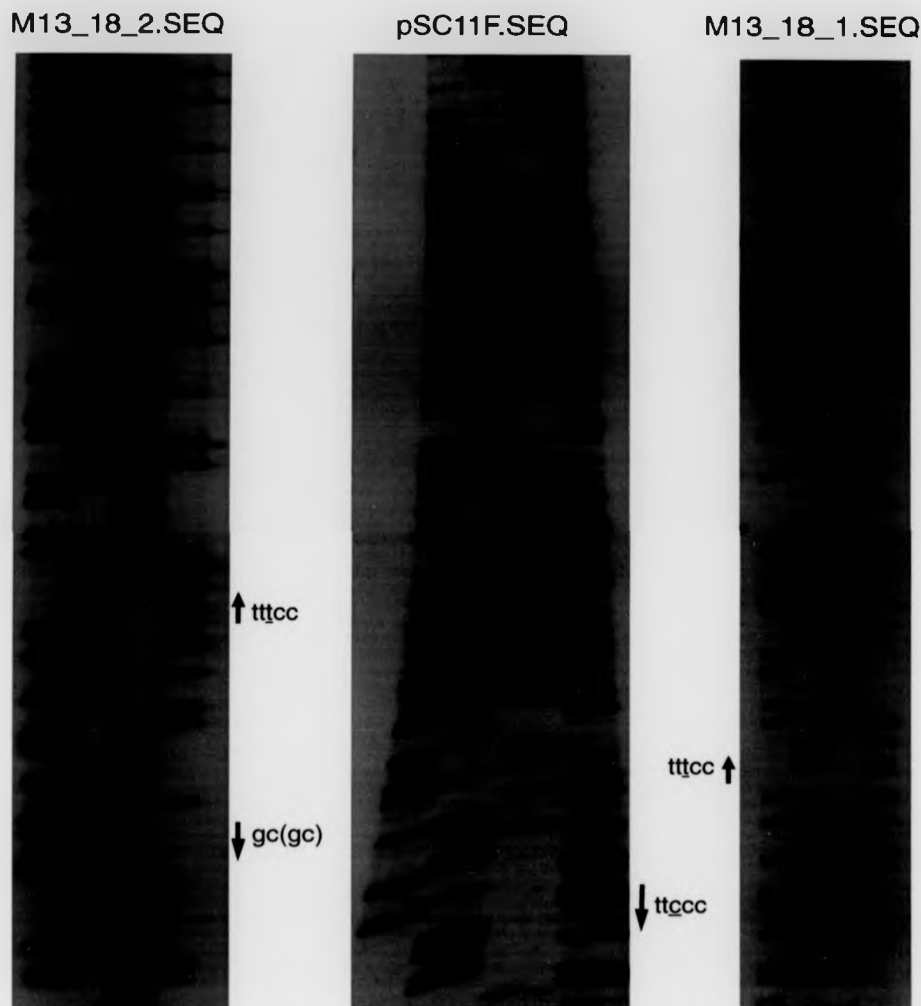


Figure 3.4 pSC11-ESAT-6 sequencing gels

Three sequence runs are shown demonstrating data derived from double-stranded (pSC11F.SEQ) and single-stranded sequencing (M13_18_1.SEQ and M13_18_2.SEQ) - see Figure 3.2 for details of the runs. A 'C to T' substitution is shown in two runs from the same M13mp18 clone (upward arrows). The correct sequence is indicated in the double-stranded sequence data (downward arrow, middle image) and was confirmed in 2 other M13 clones. One of several GC-rich compressions is shown in the left hand image (downward arrow) where 'gc' should read 'gcgc' (positions 71-74). All of these were resolved by sequencing in the other direction, using an M13mp19-ESAT-6 clone.

contamination and was not used in further experiments. Further optimisation was achieved by infecting near confluent monolayers rather than 80% confluent monolayers, using low passage TK-143 cells obtained from Oxford (Dr T. Blanchard) and more powerful cup sonication (Section 2.14.2). With these methods, a viral yield of between 5×10^7 and 10^8 pfu/T175 monolayer was obtained.

3.2.4 Verification of rVV-ESAT-6 at the genome level

3.2.4.1 PCR verification

Viral genomic preparations were made as described in Section 2.16.1.1. These were used as template in the standardised *esat-6* PCR protocol (Section 2.9.3). Both purified MTB genome and the *esat-6* cloning vector, pAA249, were used as positive controls (Figure 3.5a). A clear band of the predicted size (292 bp) was seen with the rVV whether the template was used neat or diluted 1 in 10. A signal of the correct size was present with the undiluted MTB genome preparation, but disappeared when the template was diluted 1 in 10. A similar PCR reaction was carried out using BCG genome, and, as predicted from the known deletion of the *esat-6* gene (Mahairas et al. 1996), a signal was not seen (data not shown).

3.2.4.2 Verification using Southern blotting

A whole gene digoxigenin-labelled probe and chemiluminescent protocol was employed (Section 2.16.1). In order to check uptake of the label, the probe was dotted onto nylon membrane and the labelling checked as described in Section 2.16.1.4. Figure 3.5b shows successful labelling with a signal readily identified at 1 in 1000 dilution. Figure 3.6 demonstrates that *esat-6* was integrated into the VV genome, showing agarose gel electrophoresis of *EcoRI*-digested plasmids and rVV with and without the *esat-6* gene insert and the corresponding Southern blot probed with the digoxigenin-labelled *esat-6* probe. The gel electrophoresis shows 400 bp and 700 bands of the *EcoRI*-digested plasmids in lanes 1 and 2 (Figure 3.6a), consistent with the respective predicted fragments for pSC11-ESAT-6 and pSC11 alone (see map, Figure 2.1b). In the Southern blot, the autoradiograph demonstrated strong signals for both the digested rVV-ESAT-6 genome and the positive control, pSC11-ESAT-6 (lanes 2 and 4). These bands are approximately 700 bp in size. The *EcoRI*-generated 700 bp band in the pSC11-ESAT-6 lane corresponds to the signal in the equivalent lane of the Southern blot. This corresponds to the predicted fragment size (see map, Figure 2.1b). The same map indicates that an *EcoRI* digest of whole rVV genome

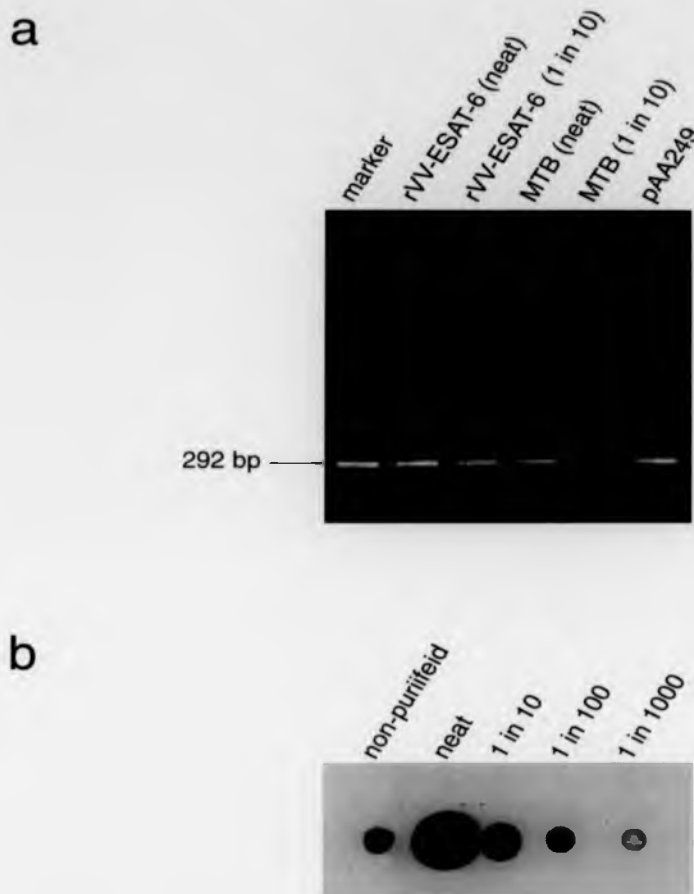


Figure 3.5 PCR verification of rVV-ESAT-6 and assessment of an *esat-6* probe
a) Integration of *esat-6* into VV was assessed using PCR amplification in conjunction with the *esat-6* cloning primers, giving a predicted product size of 292 bp. A genomic preparation of a twice plaque-purified rVV-ESAT-6 preparation was used as template at 2 concentrations (neat and 1 in 10) and the products were run in the 2nd and 3rd lanes respectively. In addition, an MTB genomic preparation, at 2 concentrations, and a plasmid carrying *esat-6* were used as PCR template positive controls (4th to 6th lanes from left). **b)** In order to verify adequate labelling of a digoxigenin-labelled *esat-6* whole gene probe, the PCR-generated probe was dotted onto a nylon membrane in a non-purified form (left dot) and in a gel and resin-purified form (neat and in 3 tenfold dilutions). The membrane was subjected to a chemiluminescence protocol (Boehringer Mannheim) and the signal recorded on ECL hyperfilm.

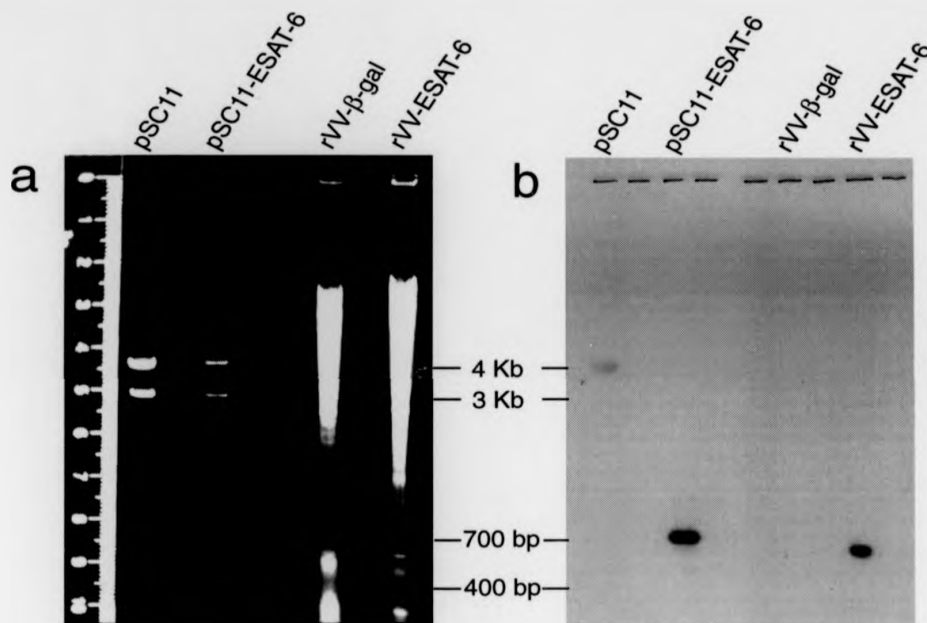


Figure 3.6 Southern blot of rVV-ESAT-6 derived *esat-6* DNA

a) *Eco*RI-digested plasmids, pSC11 and pSC11-ESAT-6 (left hand lanes), and genomic preparations from rVV-infected TK 143 cells (rVV-β-gal and rVV-ESAT-6 - right hand lanes) were run on an agarose gel. **b)** The DNA from this gel was transferred to a nylon membrane and subjected to Southern blotting using a digoxigenin-labelled whole gene *esat-6* probe and a chemiluminescence protocol (Boehringer Mannheim). The signal was recorded using ECL Hyperfilm (Amersham).

would also produce a 700 bp fragment, as the same cutting sites are transferred following recombination of the insert, the selectable marker and their promoters. rVV- β gal and pSC11 alone, used as negative controls, gave no signal. The faint band in the first lane (pSC11-ESAT-6) represents background signal from the strong 4 kb band (see agarose gel). It was noted that the band from the whole genome digest was slightly advanced and narrower than the plasmid positive control. A likely cause is a gel artifact created by running more DNA in the whole genome lane (cellular and viral genome) compared with the plasmid lane.

3.2.5 rVV-ESAT-6 expression

3.2.5.1 Immunoblotting

Protein was extracted from rVV-infected TK⁻ 143 cells and quantified as described in Section 2.16.3.3. The rVV-ESAT-6 cell lysate yielded a protein concentration of 1.9 mg/ml and the ST-CF positive control was supplied at a concentration of 2 mg/ml. A minigel system was used, which permitted the loading of a maximum of 30 μ l per lane (22.5 μ l of cell lysate plus 7.5 μ l of x4 loading buffer). Following protein transfer and probing with the mAb, HYB76-8, no signal could be detected from rVV-infected cell lysates, whilst there was a strong signal from the ST-CF as demonstrated in Figure 3.7a. In an attempt to improve sensitivity, a much larger volume of lysate was loaded (150 μ l per lane) using a larger gel system and a 3 mm thick 18% polyacrylamide gel. rVV-ESAT-6 lysate was loaded neat (lane 1) and at 1 in 5 and 1 in 10 dilutions (lanes 2 and 3 respectively). Given the strong signal from the previous Western blot, the ST-CF was diluted and 3, 0.3 and 0.03 μ g total protein used for lanes 4, 5 and 6 respectively. After a 15s film exposure, no signal was present from the rVV cell lysate lanes and a strong signal was seen from the positive control (lowest dilution). Deliberate over-exposure (5m) produced a very high background, but also a signal from the second ST-CF dilution lane (0.3 μ g), marked by an arrow on Figure 3.7b(ii). Again, no signal was obtained from the rVV-ESAT-6 sample. The background in these experiments was very high. This may have resulted from not including the blocking agent as part of the antibody diluent, a protocol step recommended by the manufacturer (Life Sciences).

3.2.5.2 RT-PCR

mRNA was harvested from three separate preparations of rVV-ESAT-6 infected cells and one preparation of rVV- β gal as described in Section 2.16.2.1. Aliquots of 2 μ l were

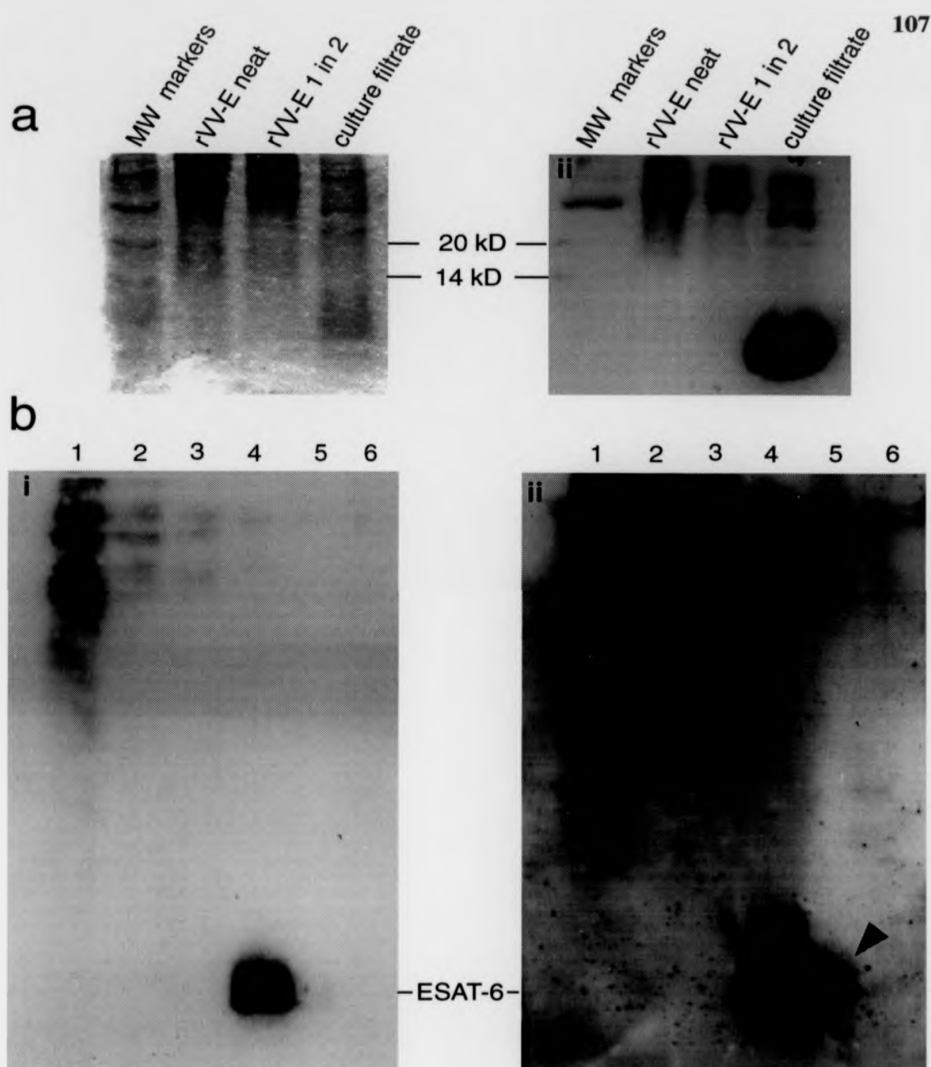


Figure 3.7 Western blots of rVV-ESAT-6 infected cell lysate

rVV-ESAT-6 infected cell lysate and MTB CF (positive control) were subjected to SDS-PAGE using small and large gel formats. These were immunoblotted and the signal detected using a chemiluminescence technique (ECL, Life Sciences). **a)i** A Coomassie blue stain of an SDS-PAGE gel is shown, which included two concentrations of rVV-ESAT-6 cell lysate (rVV-E), loaded in 10 μ l volumes per lane (neat and diluted 1 in 2). **ii** A Western blot of a replicate gel to **a)i**. The blot was probed using the ESAT-6 specific mAb HYB76-8. **b)i** A larger scale Western blot was performed using the same rVV-ESAT-6 cell lysate, but the lanes were loaded with 150 μ l, in 3 tenfold dilutions, equivalent to 285, 57 and 29 μ g of total protein respectively. Lanes 4 to 6 show the MTB CF with 3, 0.3 and 0.03 μ g, of total protein loaded in each lane respectively. **ii** This image shows an overexposed version of **b)i**. The arrow denotes presence of signal consistent with ESAT-6 (lane 5).

electrophoresed to exclude degradation (Figure 3.8a). Spectroscopy was also performed which identified preparation 3 as the most pure ($OD_{260}/OD_{280} = 1.92$). This preparation had a calculated mRNA concentration of 129 ng/ μ l. The rVV- β gal preparation had an $OD_{260}/OD_{280} = 2.01$ and a concentration of 3.04 μ g/ μ l. Generation of cDNA and PCR amplification was performed as described in Section 2.16.2.2. ESAT-6 specific mRNA transcription was readily identified using RT-PCR (Figure 3.8b). This figure also shows the relevant controls: with and without DNase treatment of mRNA preparations and with and without an RT step. These controls were used to exclude the possibility of amplification of the *esat-6* gene from contaminating viral genome in the mRNA preparations. This would have occurred if DNase treatment had not been used, as demonstrated by the faint signal seen in lane 5 where the mRNA preparation was not treated with DNase (potential for contaminating DNA) and no RT step performed (no cDNA generated). In order to exclude the possibility of a similar sized band being amplified from a rVV lacking the *esat-6* insert, PCR was also performed using four tenfold dilutions of cDNA template each derived from rVV-ESAT-6 and rVV- β gal. Figure 3.8c shows a single discrete band, visible in the first two template dilutions of rVV-ESAT-6. Only multiple bands were visible for rVV- β gal.

3.2.5.3 Optimising the immunoblotting conditions

High backgrounds were seen in the early immunoblots (Figure 3.7). Therefore, 5% milk powder in PBS-T was added to the primary and secondary antibody incubations (an accepted deviation from the recommended protocol from Life Sciences). Dot blots were performed to compare the presence or absence of blocking agent in the antibody diluent. The addition of blocking agent produced no loss of sensitivity and a greatly reduced background (data not shown). This modified protocol was then performed for Western blotting of ESAT-6 using protein samples from eight twofold dilutions of *M. marinum* culture filtrate (CF) (Figure 3.9b). This figure shows a clear signal of correct size for ESAT-6, down to lane 6 (1 in 32 dilution). This lane contained 0.16 μ g of CF protein of which <1% would contain ESAT-6 (Dr P. Andersen, personal communication) indicating that this assay had a sensitivity of ≤ 1.6 ng. In a separate experiment, a direct comparison using dot blots showed that, if anything, the signal to noise ratio was slightly improved by the addition of blocking reagent to antibody incubations (data not shown). Of note, the addition of blocking reagent did prolong the exposure time from about 30s to several minutes, with the optimum exposure for weak signals being about 15-30m.

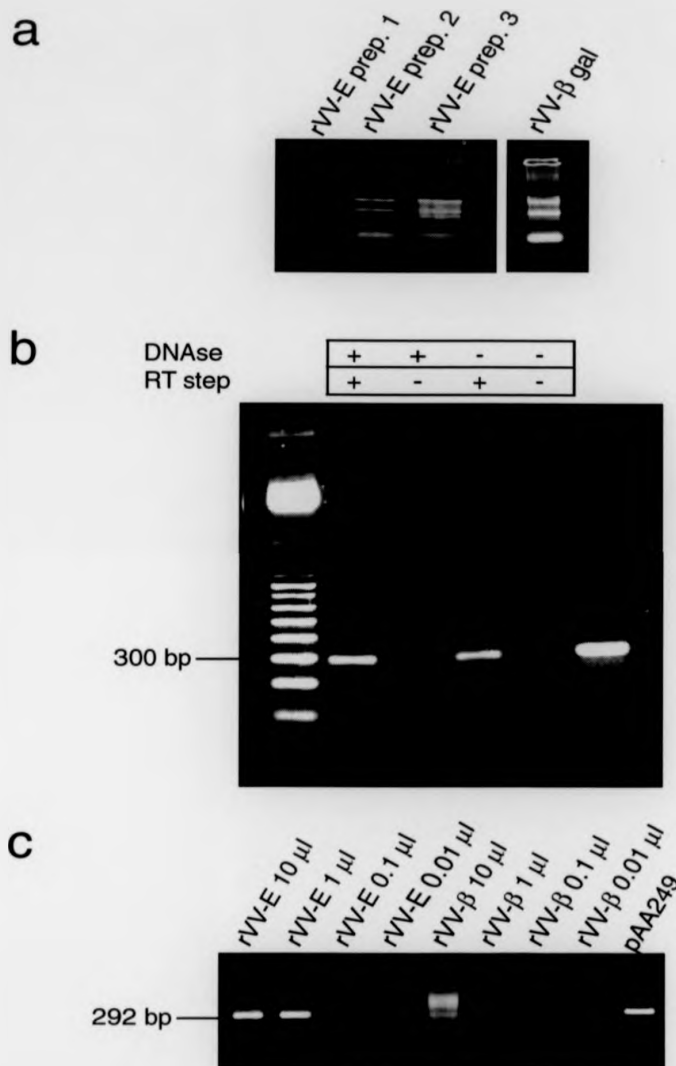


Figure 3.8 RT-PCR of rVV-ESAT-6

a) Agarose gel electrophoresis of three RNA preparations of rVV-ESAT-6 and one of rVV- β gal infected cell lysates is shown. **b)** Following reverse transcription of 'rVV-E prep. 3', PCR was performed using the *esat-6* cloning primers (predicted product size of 292 bp) and the products run as shown: lane 1 - 100 bp molecular weight marker; lane 2 - test sample (RNA sample pre-treated with DNase prior to the RT step); lanes 3 to 5 - the relevant controls (with/without DNase treatment and with/without the RT step); lane 6 - the positive control (pAA249 plasmid template which carries *esat-6*) **c)** RT-PCR was performed as for **b)**, but included rVV- β -gal (rVV- β) as an additional negative control. Reactions were replicated to include 4 tenfold dilutions of the cDNA templates.

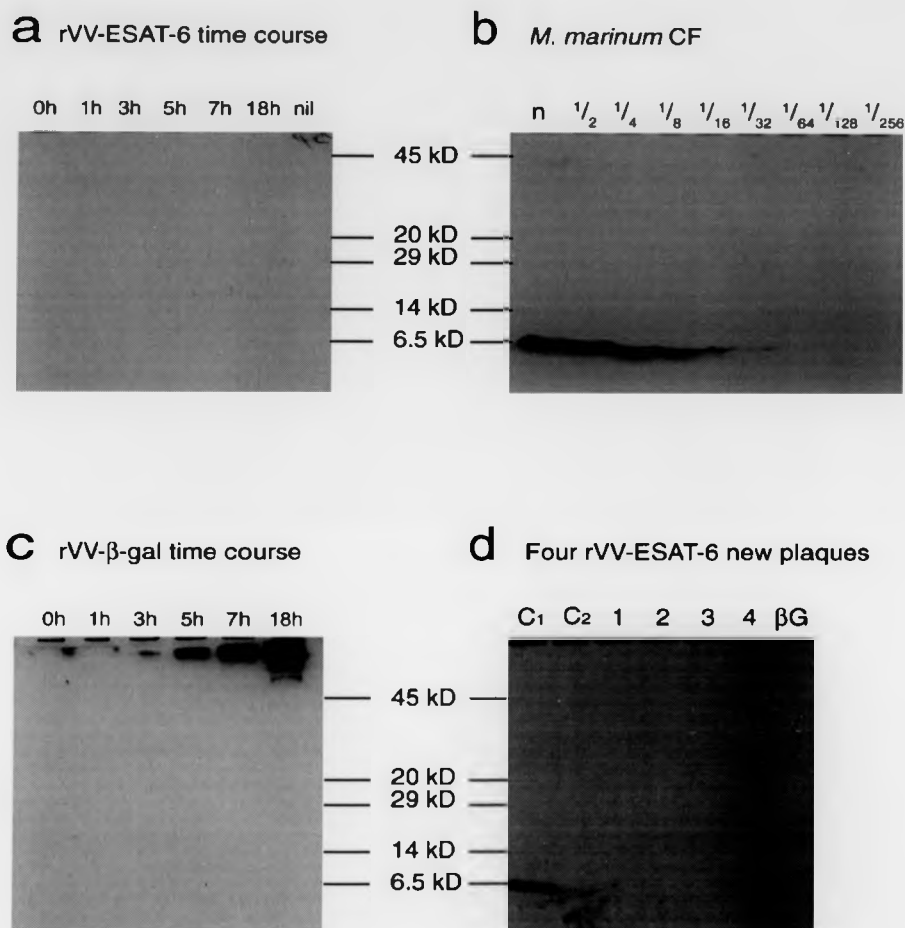


Figure 3.9 Western blots of rVV-ESAT-6 and rVV-β-gal cell lysates and *M. marinum* culture filtrate

Additional Western blots were performed in order to address possible explanations for failure to elicit a signal when probing for rVV-generated ESAT-6. **a**) A rVV-ESAT-6 time course was performed, where cell lysates were harvested between 0 and 18 hours pi and a Western blot was performed using the ESAT-6-specific mAb, HYB76-8. **b**) *M. marinum* culture filtrate (CF) was subjected to Western blotting using neat CF protein (5 μg) and a series of twofold dilutions. **c**) A rVV-β-gal time course was performed as for **a**), but was probed with an anti-β-galactosidase antibody (Boehringer Mannheim). **d**) Four rVV-ESAT-6 plaques were selected from the initial recombination mix and rVV-infected cell lysates were prepared and Western blotted (labelled 1-4). The 2 left hand lanes show *M. marinum* CF in two concentrations (C1 = neat and C2 = 1 in 10). The right hand lane (labelled βG) shows an rVV-β-gal negative control.

3.2.5.4 Excluding methodological reasons for lack of signal

- 1) To exclude the possibility that ECL immunoblotting might be inhibited by rVV-infected cell lysates, the rVV- β -gal lysate was probed with a specific anti- β gal mAb. Lysates were collected at six time points over 18h (see Figure 3.9c), proteins were resolved and blotted as described in Section 2.16.3. Figure 3.9c shows that β -gal specific bands were visible from 3h post infection. Thus, the system was easily able to detect a rVV-encoded recombinant protein.
- 2) To exclude the possibility of transient expression, a rVV-ESAT-6 time course similar to rVV- β -gal was performed. No ESAT-6 specific signal was identified at any time point, showing that expression was not transiently detectable and missed because of the previous time-point selected for sampling (Figure 3.9a).
- 3) To exclude the possibility of having selected an aberrant plaque during the purification steps, four additional plaques from the original recombination mix were picked and grown up. No signal was seen when probing these cell lysates for ESAT-6 (see Figure 3.9d). This confirmed that the lack of signal was not due to an error in the rVV clone selected at the outset.

3.3 DISCUSSION

rVV-ESAT-6 was successfully constructed using the pSC11 insertion plasmid and standard techniques. This represented the first known rVV to incorporate the *esat-6* gene. Fidelity of cloning was verified by gene sequencing, PCR, Southern blotting and RT-PCR. However, repeated immunoblotting with alteration of several parameters failed to identify protein expression. Eighteen months later, following the development of a capture ELISA by Prof M. Harboe, rVV-ESAT-6 protein expression was identified (Figure 5.7) (Harboe et al. 1997). This will be discussed in chapter 5.

3.3.1 Why was protein expression not identified?

Following rVV-ESAT-6 construction both Southern and Western blotting were performed. These data showed viral incorporation of the gene but no gene expression using the only available antibody (at that time, no polyclonal sera or alternative mAbs existed). Two explanations amenable to investigation were addressed.

3.3.1.1 Potential misincorporation during PCR insert generation

In the early stages of the study, plasmid cloning had not been optimised and the results of T:A cloning were poor. Ten ligation/transformation experiments and over a hundred minipreps were required to obtain one successful clone. This was in marked contrast to the optimised cloning subsequently developed and described in chapter 4. In later experiments, *Taq* rather than *Pfu* polymerase was used. This enzyme permitted the omission of the A-tailing step (Figure 3.1c) and resulted in a higher insert yield. However, *Taq* polymerase does not possess 3' proof reading activity and the potential for PCR misincorporation is approximately 10 fold greater than with some of the high fidelity DNA polymerases such as *Pfu* (Lundberg et al. 1991). The error rate (ER) for *Taq* has been calculated at 2×10^5 per base pair per cycle (Eckert and Kunkel, 1990), whilst for *Pfu* the rate is 1.6×10^6 (Lundberg et al. 1991). Thus, using the equation:

$$\% \text{ error free amplification} = \left((1 - \text{ER})^{\text{no. of bp}} \right)^{\text{no. of cycles}} \times 100$$

For *Taq*, the % error free amplification is 86.4%

For *Pfu*, the % error free amplification is 98.8%

(where the number of base pairs = 292 and the number of cycles = 25)

It should be noted that these error rates have been obtained empirically and may vary according to a number of factors including: the template, primer pair, dNTP and Mg concentration, and pH (Lundberg et al. 1991). However, despite the relatively high chance of misincorporation using *Taq* polymerase, PCR fidelity was confirmed by sequencing *esat-6* forwards in pSC11-ESAT-6 and in both directions in M13 subclones (Figure 3.2).

3.3.1.2 Potential failure of foreign gene transcription

A vaccinia promoter is necessary for viral transcription of foreign gene inserts. The virus uses its own multisubunit RNA polymerase, an exception being the T7 hybrid system where the foreign insert is under the control of the T7 polymerase promoter. A motif encoding transcriptional termination for vaccinia early genes has been recognized (5'-TTTTTNT-3') (Mackett, 1995) but this was not present in *esat-6*.

As with other vaccinia promoters, it is believed that transcriptional initiation occurs within the p7.5 promoter region. It is recommended that the ORF of the foreign insert is cloned as close as possible to the viral promoter (Mackett et al. 1982; Mackett et al. 1984). The cloning of this construct used a PCR-generated insert of the ORF without the addition of 5' untranslated sequence. Despite these measures, it was still possible that for some idiosyncratic reason, the gene was not transcribed. Therefore, RT-PCR was carried out. This demonstrated that failure of transcription was not the cause of absent gene expression (Figure 3.8).

3.3.1.3 *Rapid degradation of nascent recombinant protein*

ESAT-6 is a small protein of prokaryotic origin. In its native state, it is actively secreted by some strains of mycobacteria by a signal peptide independent process (Harboe et al. 1996). Whatever mechanisms permit protein stability and secretion would not necessarily apply in a mammalian cell. Indeed, it is possible that its presence in the eukaryotic cytoplasm could give rise to rapid proteolysis, thus preventing identification of the whole protein. However, one could assume that protein degradation would give rise to MHC class I antigen processing and presentation of ESAT-6 peptides to CD8+ T cells. Thus, a CTL assay could be used as a readout for protein expression, providing there was an adequate system for restimulating low precursor frequency ESAT-6 specific CD8+ CTL. However, the methodology available in our laboratory used live BCG for T cell restimulation, which lacks ESAT-6. Eighteen months later, collaborators in Oxford (Prof A. Hill's group) demonstrated class I restricted, CD8+ T cell recognition of this rVV-ESAT-6 using peptide restimulation of human CTL (see chapter 6) (Lalvani et al. 1997).

3.3.1.4 *Further potential experiments not performed in this study*

Immunoblotting was limited, owing to the fact that only a single mAb, specific for ESAT-6, existed at the time of experiments. Alternative strategies to identify protein production could have included direct identification using immunoprecipitation or immunofluorescence (offering the potential of increased sensitivity) (Mackett, 1995). In addition, indirect identification may have been possible using: (i) IFN- γ production or T cell proliferation to rVV-infected cell lysate using mouse immune splenocytes (Andersen et al. 1991); (ii) animal immunisation with rVV-ESAT-6 and testing for a recall response with ESAT-6 from a source different from rVV; and (iii) immunoblotting the rVV-ESAT-6 cell lysate using sera from animals immunised with ESAT-6 protein.

3.3.2 Which way forward? Pitfalls of CTL

Performing CTL experiments to recognise MHC class I associated ESAT-6 peptide represented the most likely chance of obtaining a positive signal from the ESAT-6 construct. At this stage in the study, it was felt that there were significant problems in pursuing this approach. These are discussed below.

3.3.2.1 A whole antigen approach

As stated previously, no immediate means for CTL restimulation were available. Whilst BCG did restimulate CD8⁺ T cells, the organism does not include ESAT-6. As an alternative, virulent MTB could have been used, but we had little experience of working with this Category III organism in tissue culture. *Mycobacterium marinum* represented a source of ESAT-6 (the only Category II *Mycobacterium* spp. expressing this protein), but it was not known whether this would reproduce the CD8⁺ CTL restimulation property identified using live BCG (Turner and Dockrell, 1996).

3.3.2.2 A peptide approach

Using peptides to restimulate CTL represented an attractive approach, but not without drawbacks. The main problem would have been identifying which peptides to use. Overlapping peptides could miss important peptides, with the potential for missing a key peptide straddling a region represented by two peptides. Alternately, predicted peptides based on HLA class I specific motifs, could have been used in conjunction with knowledge of HLA resistance alleles. Hill and colleagues (1992) successfully used this technique for the identification of malaria CTL peptides and HLA B53. However, this 'reverse immunogenetics' requires knowledge of an HLA resistance allele, and these data are not available for TB (Pitchappan, 1990). Another option was to scan for predicted peptides for common HLA alleles within the sequence of *esat-6* and screen using a class I binding assay or assess for CTL restimulation using pools of peptides. This represents a useful approach. However, problems can arise whereby peptide-restimulated CTL can be generated to kill peptide-pulsed target cells, but not target cells expressing endogenously processed whole antigen (Stauss et al. 1992). This may be a consequence of differential processing by antigen presenting cells (Eisenlohr et al. 1992). Furthermore, alternative peptides not predicted by known algorithms can also induce CTL (Sadovnikova et al. 1994). From a logistic perspective, the number of donors would be restricted. Each donor would require HLA

typing with selection of only those with an HLA type corresponding to the available panel of peptides.

3.4 CONCLUSION

A strategic decision was made to pursue construction of further rVV rather than look for CTL using the available rVV-ESAT-6. The pursuit of CTL at this stage was felt to be high risk given the difficulties in restimulating biologically-relevant ESAT-6 specific CTL. In addition, whilst identification of ESAT-6 specific CTL would have been beneficial, failure to find CTL would have begged the question of a problem with the construct rather than absence of significant CTL in the subject population. Therefore, several strategies were pursued in order to maximise the chances of obtaining rVV with identifiable expression of MTB secreted proteins.

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4.1 INTRODUCTION

Failure to identify protein expression from rVV-ESAT-6 led to three alternative strategies: (i) the use of genes for three further *M. tuberculosis* secretory proteins; (ii) the addition of a signal sequence; and (iii) the application of the vaccinia/T7 hybrid system. This chapter describes construction of two vaccinia insertion plasmids containing a signal sequence derived from tPA (p1108-tPA and pWR510-tPA), PCR generation of the required genes prior to cloning, development of rVV and verification of homologous recombination at a DNA level. Studies of rVV protein expression are included in chapter 5.

4.1.1 Genes encoding additional MTB secreted proteins

4.1.1.1 The choice of available genes

It was decided to use additional secreted proteins for *M. tuberculosis*. However, the number of candidates was limited to the known available sequences. At that time, potential candidates could have included genes encoding: the Ag85 complex (Borremans et al. 1989; Content et al. 1991), the 23 kDa superoxide dismutase protein (Zhang et al. 1991), the 38 kDa phosphate binding protein (Andersen and Hansen, 1989); and the 10 kDa heat shock protein (Baird et al. 1989). As discussed in Section 1.4.3.2, the Ag85 complex represented the most attractive group of immunodominant secreted proteins. Furthermore, data presented at a Keystone meeting on Mycobacterial Disease held at Tamron, Colorado, 1995

suggested that these antigens offered protection when vaccinated as DNA constructs (Drs K. Huygen and J. Content, personal communication). Similar to rVV, DNA vaccination is known to induce CD4+ and CD8+ immunity (Huygen et al. 1996). A collaborative link was established with this group and an agreement was made to exchange reagents. If rVV were produced, these would then be used for mouse immunology studies in Brussels and human immunology studies in our laboratory.

4.1.1.2 The addition of a signal sequence to enhance expression

Whilst rapid degradation of protein in the cytoplasm would be beneficial for inducing MHC class I restricted immune responses, complete absence of identifiable expression would make the interpretation of such a negative result difficult. If a CD8+ CTL clone against a commonly recognised MTB secreted protein epitope was available, this could be used as a yardstick to assess putative CTL precursor frequency in TB patients or BCG vaccinees. Unfortunately, such CD8+ clones were not available. Therefore, one of the strategies employed was to improve the potential for protein expression by including a eukaryotic signal sequence.

Previous work had identified the value of the human tissue plasminogen activator signal sequence in a mammalian cell expression system (Chapman et al. 1991) and this had been used successfully with rVV (Wang and Mullins, 1995). Moreover, the DNA vaccine studies using Ag85 genes demonstrated enhanced immunogenicity with tPA, as compared with either no signal sequence or the mycobacterial native signal sequence (Dr J. Content, personal communication). These data led to the decision to generate rVV using Ag85 coding sequences without the native signal sequence. In addition, half the constructs would also include tPA. This would enable direct comparison of rVV both with and without this eukaryotic signal sequence.

4.1.1.3 T7 constructs

It was possible that failure to observe protein expression in rVV-ESAT-6 was a quantitative problem in that expression may have occurred, but at a level below the sensitivity of the immunoblotting. Encoding foreign genes under a late vaccinia promoter could potentially resolve this problem, where expression levels may be increased tenfold or greater (Mackett, 1995). However, during late vaccinia infection, some of the MHC class I peptide complex sites may be blocked; this is an idiosyncratic finding not seen for early antigen class I

processing and CD8+ CTL recognition (Browne et al. 1988). Furthermore, Broder and colleagues (1994) provide data showing that vaccinia late proteins are not expressed in cultured human macrophages. In contrast, proteins expressed using the T7 hybrid system to infect macrophages resulted in very high recombinant protein expression. Therefore, it was decided to construct rVVT7, expressing each of the genes, both with and without tPA. These constructs could be used for transient expression for use of identification of CTL, but they would not be suitable for animal vaccination (recombinant protein expression requires co-infection with two vaccinia constructs, see Section 1.6.7.2). In addition, the T7 insertion plasmids required for rVV construction would permit *in vitro* transcription/translation studies and provide further data concerning the effect of the signal sequence (chapter 5).

4.2 RESULTS

4.2.1 Generation of Ag85 and ESAT-6 gene inserts

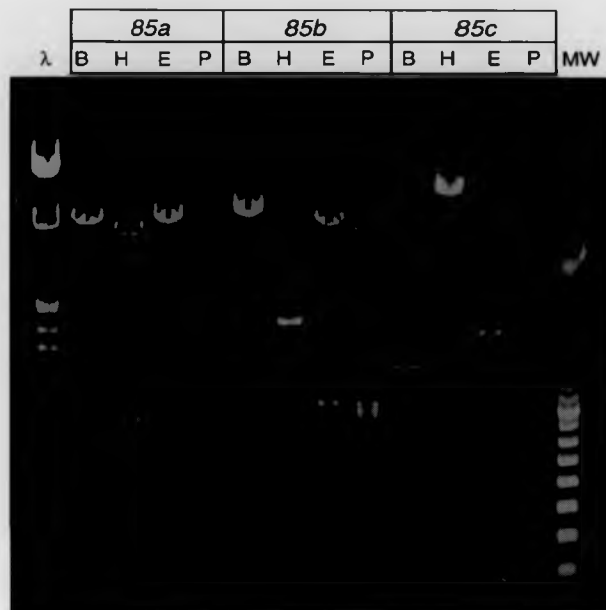
4.2.1.1 Restriction digest verification of cloning plasmids containing Ag85 genes

The three genes were obtained in plasmid form from the Pasteur Institute, Brussels (Section 2.4.2). In order to confirm their identity, restriction digests were performed based on maps from the literature (Borremans et al. 1989; Content et al. 1991). Figure 4.1a shows four enzyme digests (*Bam*HI, *Hind*III, *Eco*RI and *Pst*I) for each of the three BlueScribe (pBS) clones. The banding pattern was consistent with the restriction digest maps, thus confirming their identity.

4.2.1.2 Optimising PCR and potential pitfalls

Primer pairs were selected according to the criteria laid out in Section 2.5.3. The computer predicted primer pair efficacy gave very low scores for *85a* products when the upper primers were selected to encode the recombinant protein from the start of the mature 85A protein, where the first residue is Phe (Content et al. 1991). Examples of these upper primers include PR85A14F (includes a *Bam*HI site) and PR85A01F (includes an *Xba*I site) - see notes section in Table 2.3. When paired with an otherwise effective lower primer, PR85A15R, PR85A14F gave no signal (Figure 4.2a, lane 8). The upper primer, PR85A01F, was able to generate a product when paired with PR85A02R, but the signal was poor and a double band was generated when using *Pfu* polymerase (Figure 4.3a). The *85a* gene could be amplified using an upper primer which included an additional three or four codons upstream from the first residue of the mature protein e.g. PR85A13F, see Figure 4.2a, lane 4, and PR85A03F, see Figure 4.4b(ii).

a



b

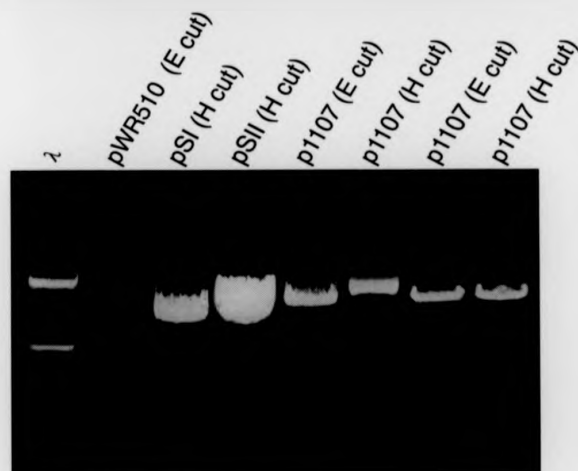


Figure 4.1 Restriction digest verification of plasmids

a) Bluescribe clones carrying one of the Ag85 genes (85a, 85b and 85c) were subjected to single enzyme digestion with four different restriction enzymes, B=*Bam*HI, E=*Eco*RI, H=*Hind*III and P=*Pst*I. b) The vaccinia insertion plasmids, pWR510, pSI, pSII, p1107 and p1108 were digested with either *Eco*RI (E cut) or *Bam*HI (B cut). All banding patterns were consistent with known restriction maps. MW=100 bp molecular weight marker. λ =lambda *Eco*RI/*Hind*III digest molecular weight marker.

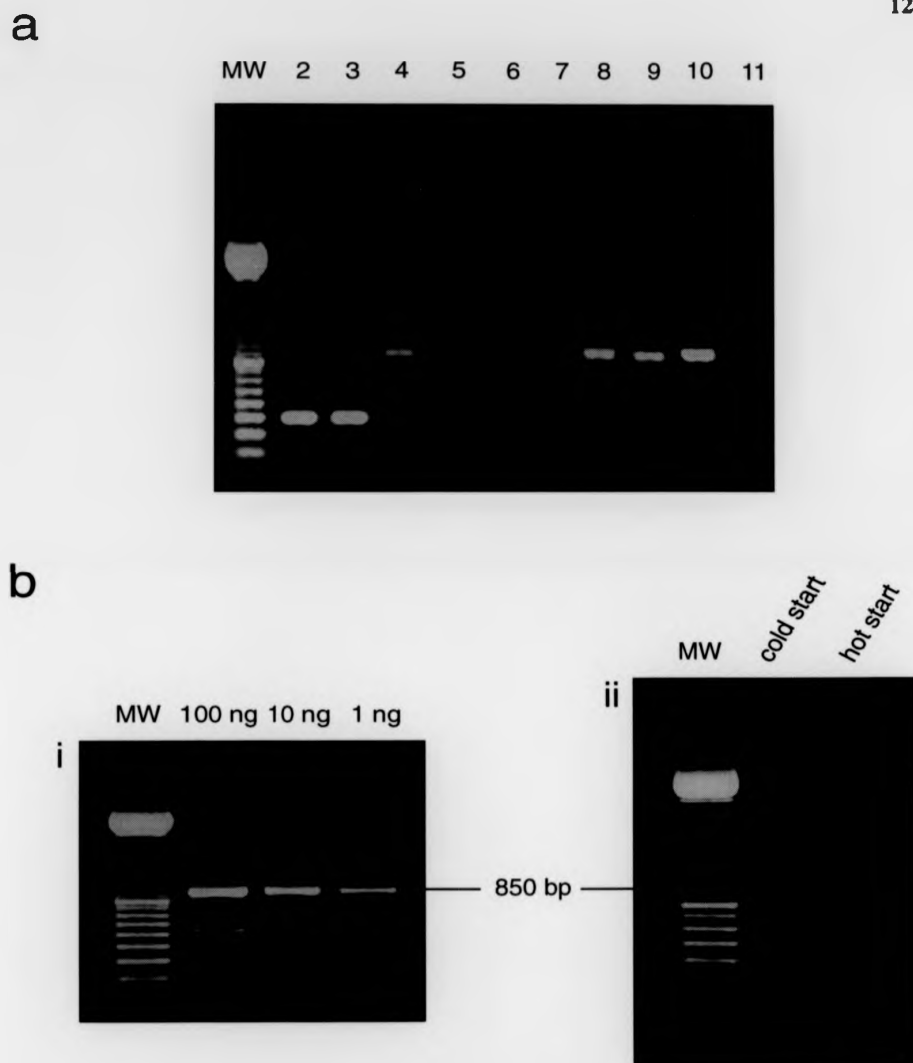


Figure 4.2 Assessment and optimisation of PCR cloning primers

a) Agarose gel electrophoresis of *Pfu* polymerase PCR products using several primer pair combinations: lane 2 17/18 (*esat-6* with *NheI*), lane 3 19/20 (*esat-6* with *BamHI*), lane 4 13/02 (*85a* with *NheI*), lane 5 14/04 (*85a* with *BamHI*), lane 6 13/04 (*85a* with *NheI*), lane 7 14/15 (*85a* with *BamHI*), lane 8 13/15 (*85a* with *NheI*), lane 9 16/22 (*85b* with *NheI*), lane 10 21/10 (*85c* with *NheI*), lane 11 03/04 (*85a* with *BamHI*). **b)i** A range of template quantities (100, 10 and 1 ng) was assessed to identify which gave the maximum yield using primer pair 01/02, pBS-85A as template and a *Pfu* polymerase PCR protocol. **b)ii** Hot and cold start PCR protocols were assessed using otherwise identical parameters. MW=100 bp molecular weight marker.

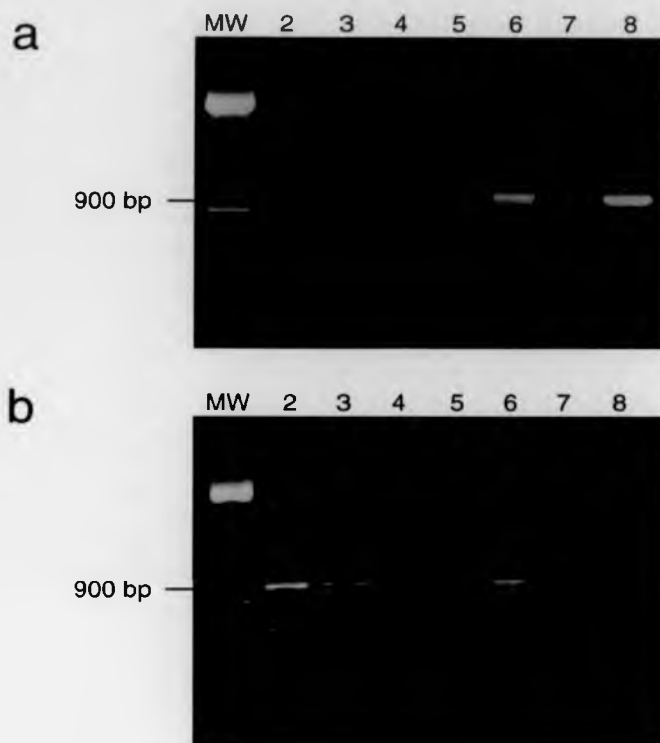


Figure 4.3 Differences in PCR amplification using *Pfu* and *Taq* polymerase

a) A variety of reaction conditions were used in order to identify the optimum means to obtain the difficult-to-amplify *85a* product when using *Pfu* polymerase and the primer pair 01/02. Lanes 2-4 represent products using the PCR cycle protocol: 98, 50, 75 (melting, annealing, extension temperatures in degrees centigrade - see text for further details). Lane 2 includes no additive, lane 3 10% DMSO, and lane 4 2% glycerol. Lanes 5-7 include products after varying the annealing temperature. Lane 5 cycle protocol = 95, 75, 55, lane 6 = 95, 75, 50 and lane 7 = 95, 75, 45. Lane 8 is the *Taq* polymerase product showing a single band (annealing temp. 50). **b)** The *Taq* polymerase product was diluted to assess if a double band was hidden within the strong signal. Lanes 2-4 represent the *Taq* polymerase product diluted 1 in 2, 1 in 5 and 1 in 10. Lanes 5-8 represent *Pfu* polymerase products showing double bands despite varying the annealing temperatures: 55, 50 and 45 for lanes 5-6 respectively and lane 8 includes the addition of 10% DMSO (annealing temp. 50). MW=100 bp molecular weight marker.

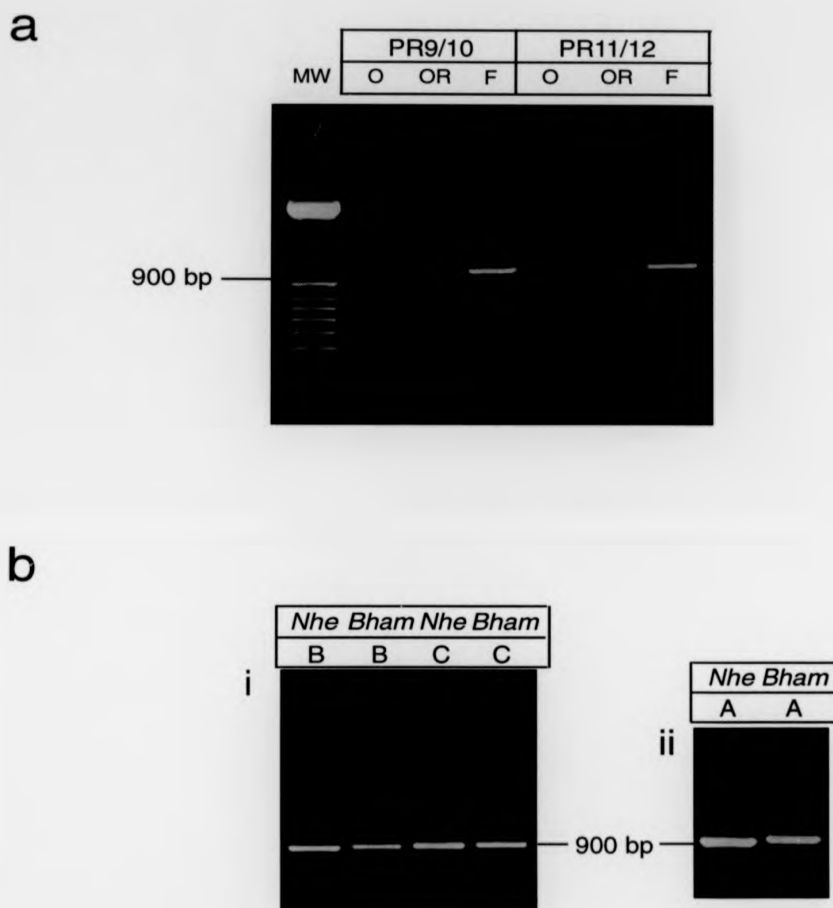


Figure 4.4 Ag85 PCR products used in cloning experiments

a) Pre-prepared primer pairs were initially stored at -20 degrees centigrade under wax pellets for bulk use. A comparison of PCR product yield was made for primers: stored (O=old), stored but reheated just prior to use (OR=old/reheated), and primers diluted and mixed just prior to use (F=fresh). **b)** Gel showing Ag85 gene inserts generated by PCR, using the cloning primers which performed the best. Primer pairs incorporated 5' restriction sites suitable for cloning into either pWR510 (*Nhe*I) or p1108 (*Bam*HI). **i** *Pfu* polymerase products for *85b* (B) and *85c* (C) genes. **ii** *Taq* polymerase products for *85a* (A). MW=100 bp molecular weight marker.

The three other genes, *esat-6*, *85b* and *85c*, were readily amplified using *Pfu* polymerase (see Figure 4.2a).

In an attempt to optimise primer efficacy for 'difficult-to-amplify' products, a number of strategies were pursued, as listed in Section 2.9.4. In the case of primer pair 01/02, a switch to *Taq* polymerase resulted in a clear signal; the intensity of the signal was proportional to the amount of template (Figure 4.2b). A signal of the same molecular weight was also produced using *Pfu* polymerase with a 'hot start', but not 'cold start' (Figure 4.2c and Section 2.9.2 for methodology). However, use of this enzyme produced a clear double band.

This may have arisen as a consequence of secondary structure within the template, although mispriming could also have occurred. Several attempts were made to manipulate the reaction conditions to allow the high fidelity DNA polymerase, *Pfu*, to be used in preference to *Taq*. These were based on the prevention of secondary structure formation using a higher melting temperature (98°C), with or without the addition of 2% glycerol or 10% DMSO. Figure 4.3a shows that none of these methods were successful. In another experiment, in order to reduce the chance of mispriming, the annealing temperature was altered, but this also failed to remove the second band (Figure 4.3b, lanes 5-7). In order to confirm that the *Taq* PCR protocol did not also produce a double band, perhaps hidden by the strong signal seen in Figure 4.2b, the *Taq* PCR product was diluted 1 in 2, 1 in 5 and 1 in 10 and the electrophoresis run time was prolonged. Figure 4.3b, lanes 2-4 confirmed the presence of a single *Taq*-generated band with an obvious double band from *Pfu* PCR for comparison.

In order to streamline the hot start PCR, batches of primer pair aliquots were stored in thin-walled PCR tubes under wax at -20°C; a standard practice carried out for MTB diagnostic work in the same department (Dr Stuart Wilson, personal communication). However, the use of these stored tubes resulted in an unacceptably low yield of PCR products. Figure 4.4a shows a comparison between stored primer pairs (O), stored but reheated to 80°C primer pairs (OR) or freshly made reaction tubes (F) for two sets of primers. It is clear that under these reaction conditions, freshly made up primers were essential for production of an adequate PCR product yield.

4.2.1.3 Inserts used in cloning

The shaded boxes in Table 2.3 indicate the primer pairs used to generate each insert used in cloning and the plasmids generated using these inserts are listed in Table 4.1 below. The optimised PCR protocol (Section 2.9.4) employed the high fidelity *Pfu* polymerase to generate all of the inserts except for the *85a* products which required *Taq* polymerase. The annealing temperature was set at 50°C for all primers, except 07/08 and 11/12, which required a temperature of 55°C. On the basis of the above experiments, PCR protocols were selected to generate inserts for cloning. Examples of the optimised reactions are shown in Figure 4.4b for Ag85 products and Figure 4.2a (lanes 2 and 3) for *esat-6* products.

4.2.2 p1108-tPA and pWR510-tPA vaccinia insertion plasmids

In order to minimise the cloning requirements for rVV-tPA-insert constructs, two generic insertion plasmids, p1108-tPA and pWR510-tPA, were prepared. Figures 2.2 (p1108) and 2.3 (pWR510) show the plasmid constructs with and without the tPA region. In each case, the multiple cloning site (MCS) is shown. Following insertion of tPA, both constructs lose their original MCS and share the MCS included in the tPA insert downstream from the signal sequence.

4.2.2.1 Construction of p1108-tPA

The protocol was followed as described in Section 2.11.1.2. Figure 4.5a shows both oligonucleotides before and after hybridisation. In addition, the right hand gel shows prepared vector in the penultimate (*Bam*HI/Klenow filled) and final stage of manipulation (*Eco*RI cut) prior to cloning. The ligation then required cloning of the blunt/*Eco*RI tPA fragment into the *Bam*HI, Klenow-filled, *Eco*RI cut vector.

An initial experiment produced only re-ligated vector (data not shown). Therefore, a second experiment was performed using a *Hind*III digested ligation mix. This site is present in the vector, but absent in the tPA construct (Figure 2.2). Five clones were selected and recombinants were screened using *Hind*III digestion. Figure 4.5b shows that clones 1.3 and 1.4 were not cut with this enzyme, consistent with successful recombination. This was verified using *Bam*HI and *Nhe*I digestion of the clone 1.3 and the vector as shown in Figure 4.5c. The *Bam*HI digest demonstrated presence of this site, as for p1108-tPA. This site would be absent from re-ligated vector lacking its MCS (a potential outcome following

Table ref. no.	Plasmid name	Unique identifier	Origin of insert (primer pair code)
#1	p1108	P18	n/a
#2	p1108-85A	P 7	PCR (03/04)*
#3	p1108-85B	P 1	PCR (07/08)
#4	p1108-85C	P 2	PCR (11/12)
#5	p1108-ESAT-6	P 8	PCR (19/20)
#6	p1108-tPA	P 12	hybridised oligo's
#7	p1108-tPA-85A	P 13	PCR (03/04)*
#8	p1108-tPA-85B	P 14	PCR (07/08)
#9	p1108-tPA-85C	P 15	PCR (11/12)
#10	p1108-tPA-ESAT-6	P 16	PCR (19/20)
#11	pWR510	P 19	n/a
#12	pWR510-85A	P 21	sub-cloned from P13
#13	pWR510-85B	P 17	PCR (16/22)
#14	pWR-510-85C	P 4	PCR (21/10)
#15	pWR510-ESAT del -6	P5	PCR (17/18)
#16	pWR510-ESAT-6	P 23	sub-cloned from P16
#17	pWR510-tPA	P 6	hybridised oligo's
#18	pWR510-tPA-85A	P 20	sub-cloned from P13
#19	pWR510-tPA-85B	P 9	PCR (16/22)
#20	pWR510-tPA-85C	P 10	PCR (21/10)
#21	pWR510-tPA-ESAT del -6	P11	PCR (17/18)
#22	pWR510-tPA-ESAT-6	P 22	sub-cloned from P16

Table 4.1 Plasmids used in rVV construction and transcription/translation studies

*=PCR insert generated using *Taq* polymerase. All others reactions used *Pfu* polymerase. Unique identifier code numbered chronologically according to date of construction, P=plasmid. ~~del~~=frameshift close to 5' end of *esat-6*. n/a=not applicable.

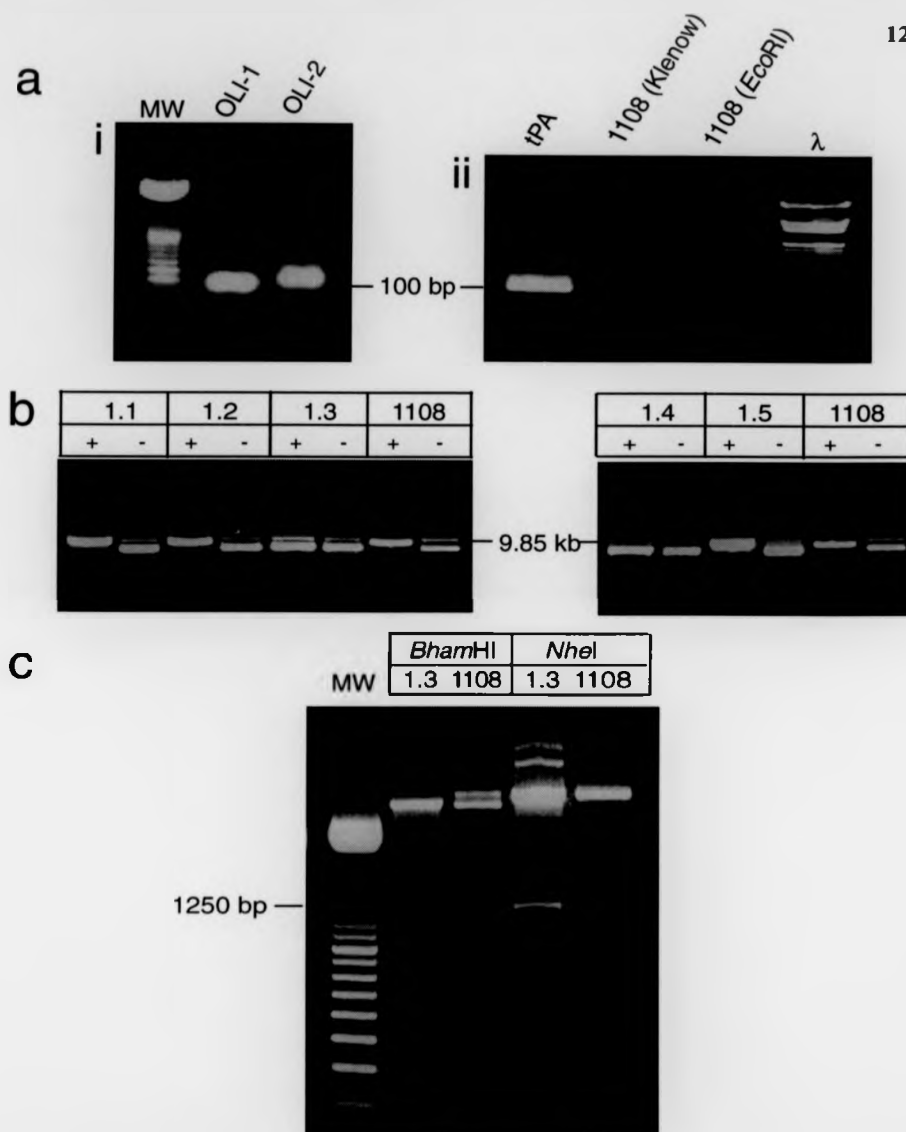


Figure 4.5 Construction and verification of p1108-tPA

a) i Agarose gel electrophoresis of tPA sense and antisense oligonucleotides (OLI-1 and OLI-2). **ii** Electrophoresis of the insert and vector prior to cloning: lane 1=hybridised oligonucleotide pair (the tPA insert), lane 2=*Sma*I digested, then Klenow filled p1108 and lane 3=*Sma*I cut, Klenow filled, then *Eco*RI digested p1108 (the fully cut vector). **b**) After cloning tPA into p1108, plasmid preparations of 5 transformants (1.1 to 1.5) were picked and subjected to *Hind*III digestion (+) or left uncut (-). Clones 1.3 and 1.4 show loss of the *Hind*III site consistent with recombination. **c**) Clone 1.3 shows preservation of the *Bam*HI site and addition of a second *Nhe*I site consistent with the predicted banding pattern for p1108-tPA. λ= lambda *Eco*RI/*Hind*III digest marker. MW=100 bp molecular weight marker.

negative selection in favour of those constructs lacking a *HindIII* site). Further support for p1108-tPA comes from the *NheI* digest which demonstrated introduction of a second site (predicted from the tPA MCS), resulting in a fragment of ~1250 bp.

4.2.2.2 Construction of pWR510-tPA

pWR510-tPA cloning was more straightforward and involved direct cloning of the tPA insert into *SwaI* cut/*EcoRI* cut vector (see Figure 4.6a). Three transformants were picked and all clones demonstrated loss of the *SacII* site, present in the vector MCS, and introduction of a *SmaI* site, present in the tPA MCS, consistent with recombination (see Figure 4.6b and map in Figure 2.3).

The MTB genes were subsequently cloned into pWR510-tPA (see below). By using sites on either side of the tPA coding region (*SwaI* and *NheI*), further evidence was provided for successful tPA recombination (see Figure 4.6c and map in Figure 2.3).

4.2.3 Gene cloning

Sixteen constructs were planned. This included all four genes in each of the four insertion plasmids, p1108 and pWR510, both with and without tPA. Directional sticky-ended cloning was employed, as described in Section 2.11.2.

4.2.3.1 A cloning example using standard methods

Following PCR amplification of five 100 µl identical reactions, the entire volume was electrophoresed and the band cut out. Figure 4.7a shows three such examples. Following electroelution, double enzyme digestion and a phenol/chloroform purification step, adequate yields of insert were demonstrated for 07/08 and 11/12 products, but no signal was seen for the 09/10 product, probably due to loss of the pellet (Figure 4.7b). This figure also shows the cut and phenol/chloroform purified vectors. In this case, *85b* and *85c* were cloned into p1108. Figure 4.7c shows a *BamHI*/*EcoRI* digest of 10 clones from the *85c* ligation, six of which showed presence of a band consistent with successful recombination. A similar result was achieved for *85b* (data not shown).

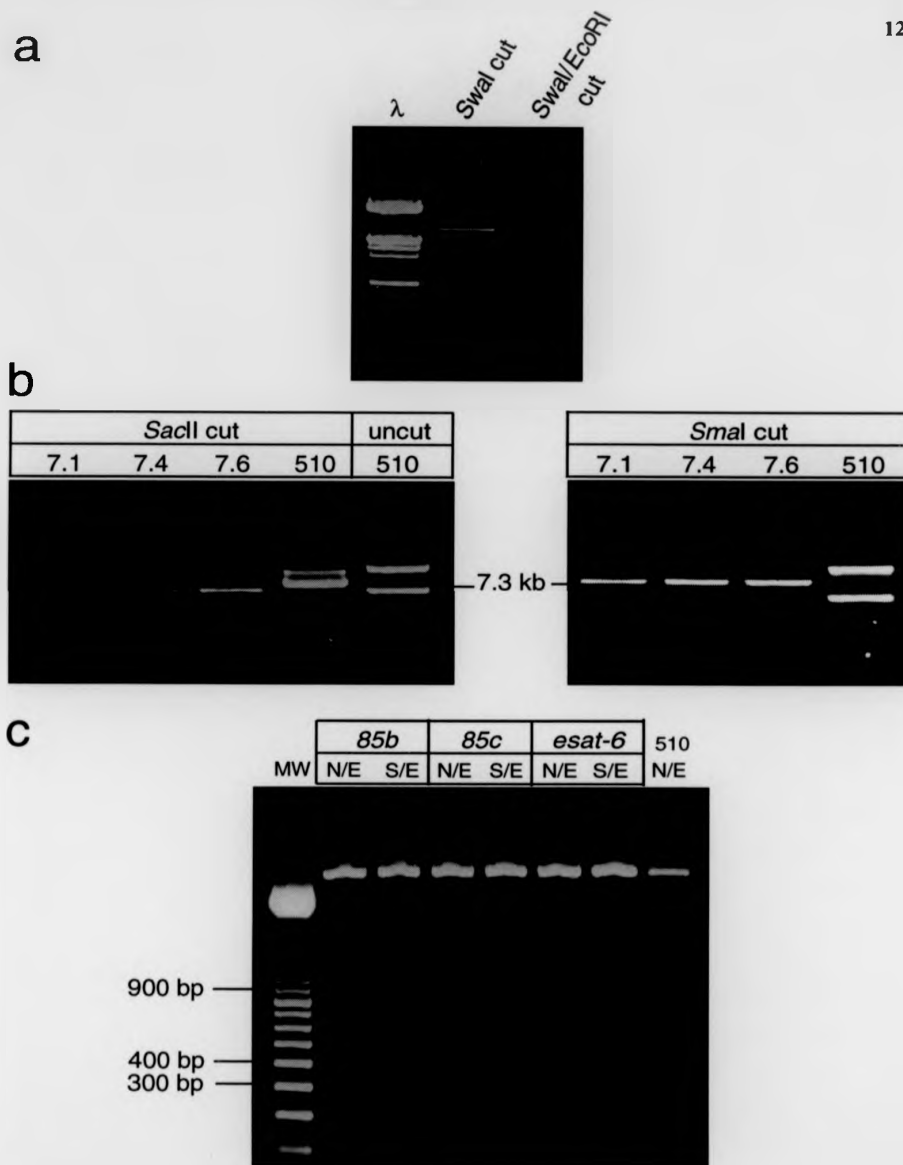


Figure 4.6 Construction and verification of pWR510-tPA

a) pWR510 was initially digested with *SwaI* (middle lane) followed by *EcoRI* (right hand lane). The tPA insert was then cloned into the *SwaI/EcoRI*-digested pWR510, three clones were picked and plasmids prepared. **b)** Two single enzyme plasmid digests were performed and the results were consistent with successful construction of pWR510-tPA for all three clones (*SacII* is present in pWR510 only and *SmaI* is present in pWR510-tPA only). **c)** Three MTB genes were cloned into pWR510-tPA and subjected to both *NheI/EcoRI* (N/E) and *SwaI/EcoRI* (S/E) double digestion. The banding pattern was consistent with correct cloning. (Furthest right hand lane=pWR510 digested with *NheI/EcoRI*).

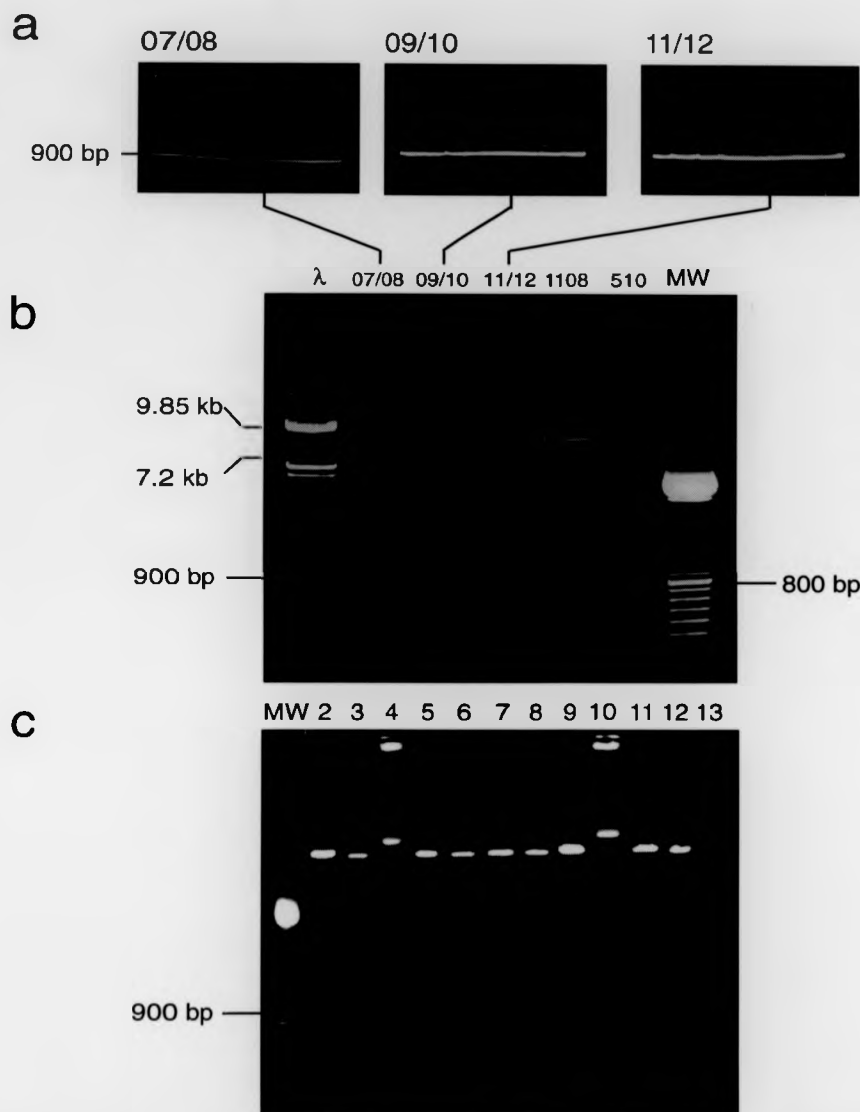


Figure 4.7 Preparation of three Ag85 inserts and the construction of p1108-85C
a) Three Ag85 PCR-generated gene inserts were purified using agarose gel electrophoresis. The primer pairs used for amplification of the inserts were: 07/08 (85*b* for p1108), 09/10 (85*c* for pWR510) and 11/12 (85*c* for p1108). **b)** Lanes 2-4 represent the gel- and phenol/chloroform-purified PCR products from **a)** which were then double enzyme digested and phenol chloroform extracted prior to cloning. Lanes 5 and 6 represent similarly prepared p1108 and pWR510 vectors. **c)** Lanes 2-11 are *Bam*HI/*Eco*RI digested clones following transformation with the p1108-85C ligation mix (primer pair 11/12). Lane 12 is *Bam*HI/*Eco*RI digested p1108 alone. Lane 13 is an 85*c* PCR product acting as a positive control. λ=lamda *Eco*RI/*Hind*III digest marker, MW=100 bp mol. weight marker.

4.2.3.2 *Microcon membrane filtration - a means to enhance cloning efficiency*

Micron membrane filtration was employed as a means to desalt both vector and insert prior to ligation. It also provided concentrated DNA suitable for cloning and separated the vector and insert from the unwanted sticky ended oligonucleotide fragments produced following restriction digestion. In addition, inserts were tested for both the removal of enzyme (Micropure EZ) and the extraction of DNA from agarose (Micropure). Figure 4.8a shows the membrane filtration system with the insert for enzyme removal and Figure 4.8b shows a gel documentation image of Micropure inserts containing ethidium bromide stained gel fragments.

High yields of concentrated, salt-reduced, cut vector and insert were obtained. For example, the gel shown in Figure 4.8a demonstrates excellent yields of fully prepared insert in a final volume of between 10-15 μ l, thus giving a working concentration of between ~50-500 ng/ μ l.

However, the same success was not achieved with the agarose gel Micropure insert (Amicon), a system designed to separate DNA from agarose without the need for electroelution or a resin-binding technique. Figure 4.8b shows very high signals at the gel purification step (left-hand gel). However, following the recommended spin time, the yields were poor (lanes 3 and 5), with much of the DNA remaining in a slurry above the membrane. A subsequent spin resulted in a similar yield, but prolonged spinning resulted in a loss of membrane integrity and loss of DNA.

4.2.3.3 *PCR screening of recombinant clones*

Initially PCR screening of clones was performed as described in Section 2.11.2.3. Once colonies had grown overnight, this screening technique simply required a single colony pick as template and a standard small volume PCR was carried out. Thus, results could be obtained within half a day. For example, Figure 4.9 shows results of PCR screening for each of the genes cloned into pWR510. Efficient recombination was achieved for three of the genes, as evidenced by clear signals of the appropriate molecular weight for *85b*, *85c* and *esat-6*, but not *85a*.

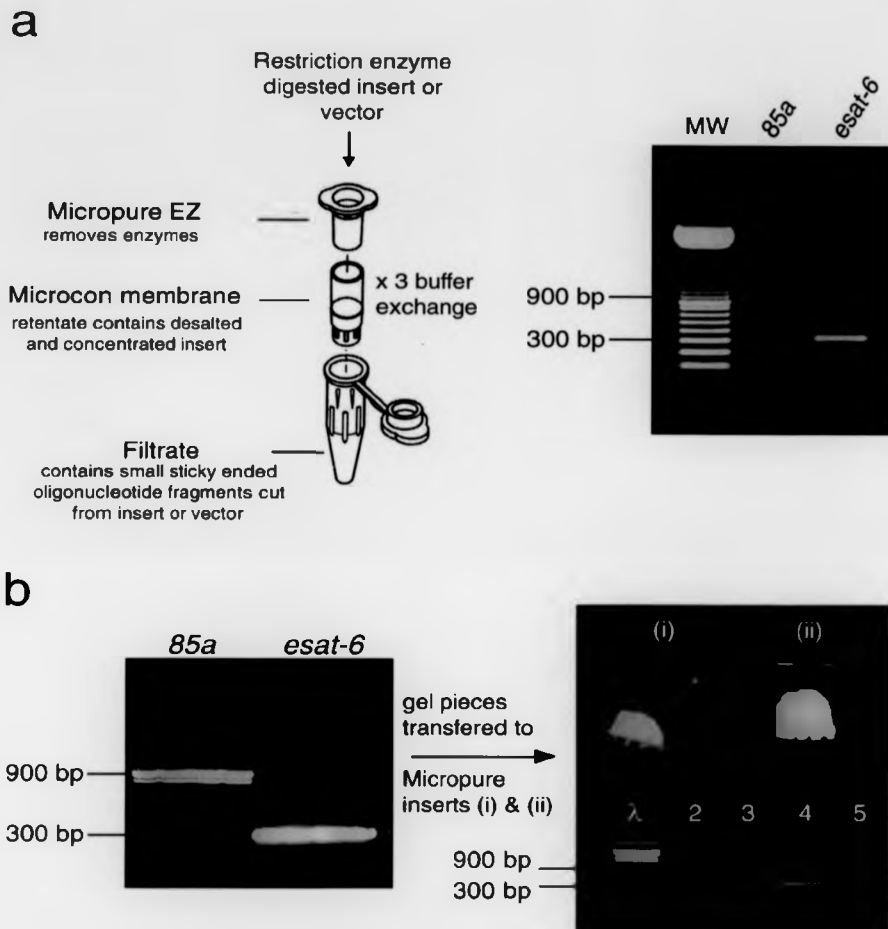


Figure 4.8 Use of Microcon spin columns to enhance plasmid cloning efficiency

a) This shows a Microcon spin column with an additional Micropure EZ plastic insert for removal of restriction enzymes. The column will remove small oligonucleotide fragments, perform a buffer exchange and concentrate the insert or plasmid prior to ligation. The agarose gel shows the yield obtained from a 500 μ l volume of PCR-generated inserts following gel- and phenol/chloroform-purification, enzyme digestion and spin column purification of *85a* (~800ng final DNA yield) and *esat-6* (~3.8 μ g final DNA yield). **b)** An assessment of the Microcon gel purification system was also carried out. Bands from preparatory gels for *85a* and *esat-6* PCR (left hand gel) were cut out and transferred to Microcon Micropure plastic inserts, (i) and (ii) (right hand gel). These were centrifuged according to the Amicon protocol. However, a gel slurry, containing most of the DNA, remained above the membrane, with only a fraction passing through for use (lanes 3 and 5). Lanes 2 and 4 represent further DNA eluted from slurry following a second centrifugation. MW=100 bp molecular weight marker. λ =lamda *EcoRI/HindIII* digest marker.

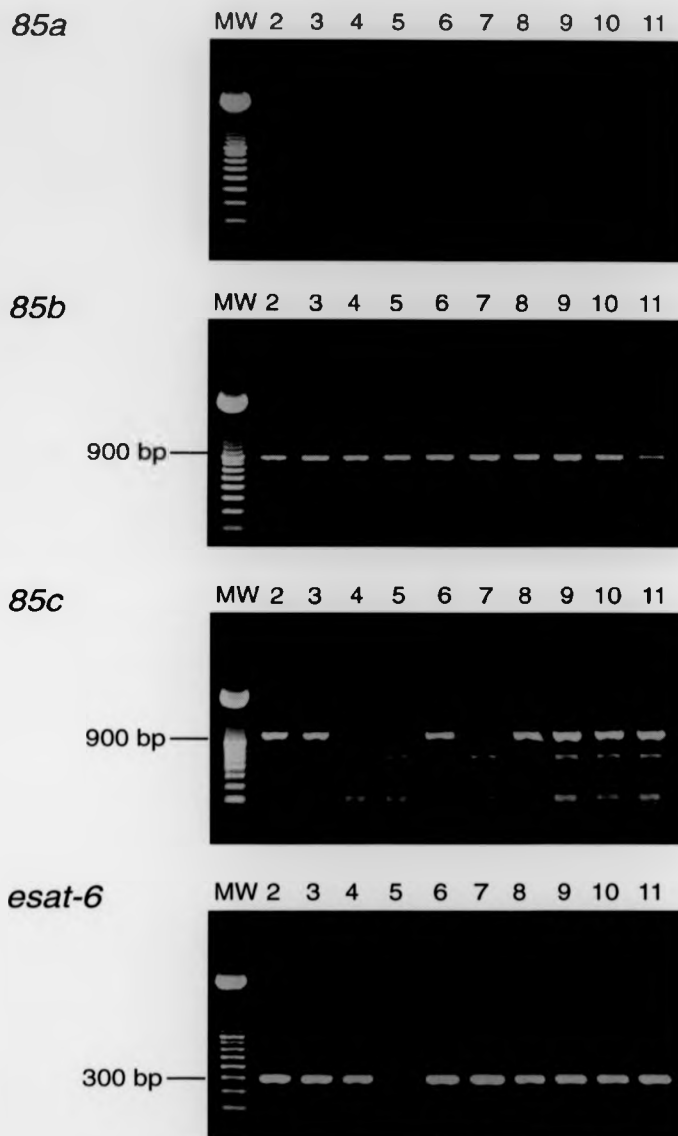


Figure 4.9 PCR Screening of pWR510 clones

Successful recombinants were identified using a PCR screening technique. Bacterial colonies were picked for each of four ligation/transformation reactions, where pWR510 was used as the vector and one of the four MTB genes was used as the insert. The agarose gels show the PCR products obtained following screening of ten pWR510 recombinant clones (lanes 2-11) for each of the four genes. The PCR template was whole *E. coli* (a 'toothpickful') and the primers were those used for generation of the insert. MW=100 bp molecular weight marker.

4.2.3.4 *Highly efficient cloning obviates the need for PCR screening*

From the PCR screening results, it was noted that using the optimised cloning strategy resulted in an 'all-or-nothing' phenomenon i.e. either the cloning was highly efficient with most clones containing inserts or the cloning failed with most clones not containing inserts. Therefore, subsequent experiments omitted PCR screening of recombinants and plasmids. Minipreps were performed selecting three to five transformants per ligation. Results of this strategy are demonstrated in Figure 4.10. Here, gels a and b show 100% efficiency for four PCR-generated inserts cloned into p1108 and p1108-tPA (all transformants selected are shown). Gels c and d represent four subcloning experiments. Genes *85a* and *esat-6* were cut from p1108-tPA and cloned into pWR510 and pWR510-tPA on a *NheI/EcoRI* fragment. Again, 100% efficiency was achieved for all 20 clones.

4.2.3.5 *Clones constructed*

Using the PCR-generated inserts, successful cloning was achieved for 14 of the 16 proposed constructs, although two of the 14 were subsequently found to contain errors (see below). Several attempts at cloning PCR-generated *85a* into pWR510 or pWR510-tPA were unsuccessful. Amplification of *85a* had been successfully achieved using either the primer pair 01/02 (*XbaI* in upper primer, *Taq* protocol, see Figure 4.4b) or the primer pair 13/02 (*NheI* in upper primer, *Pfu* amplified, see Figure 4.2a) and adequate yields of Microcon membrane-filtered cut inserts were obtained in both cases (20 ng/ μ l). However, clones screened for recombination were repeatedly negative (Figure 4.9a for 01/02, data not shown for 13/02).

Subsequently, two errors were noted in primers 06 (*85b* lower primer) and 17 (*esat-6* upper primer) (see notes column, Table 2.3). In both cases, a frame shift had been introduced in error. Therefore, *85b* was re-amplified using primer pair 16/22 and this product successfully cloned into the pWR510 plasmids. The pWR510 constructs with *esat-6* and *85a* were obtained by subcloning from p1108-tPA constructs as described above (Section 4.2.3.4, Figure 4.10).

Details of plasmid recombinants are summarised in Table 4.1. All of these plasmids were utilised in further experiments, such as rVV construction and/or *in vitro* transcription/translation. In order to improve purity, medium scale plasmid preparations (Midiprep, Promega) were performed, followed by an additional phenol/chloroform

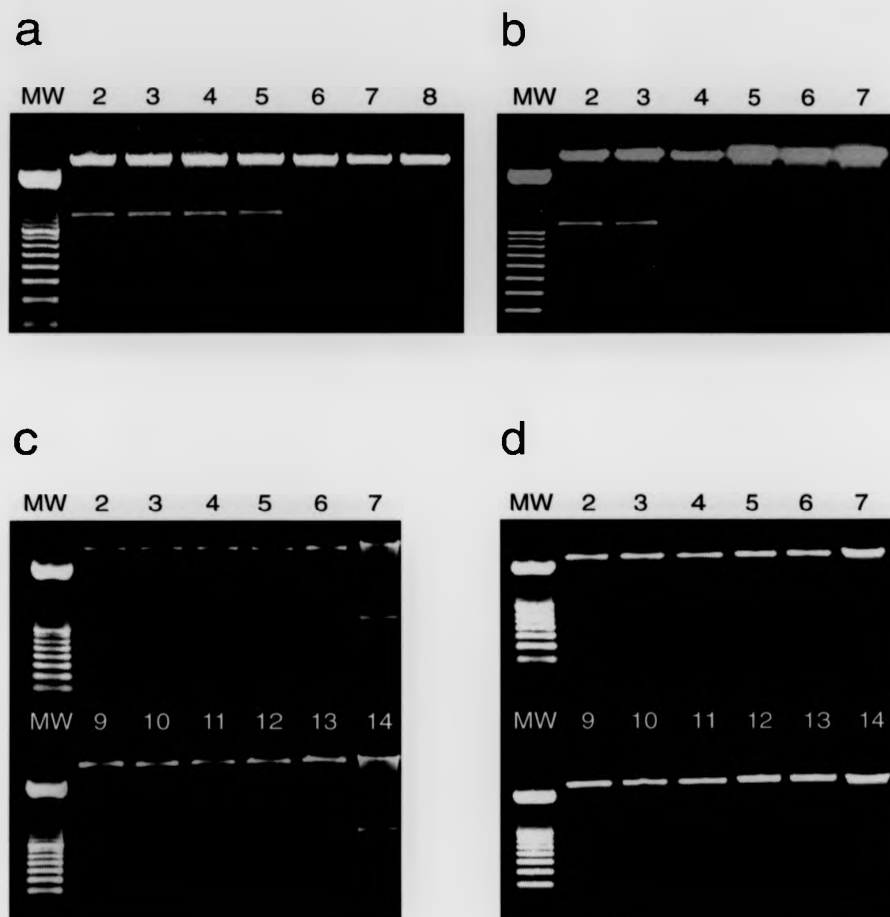


Figure 4.10 Plasmid cloning efficiency

Agarose gel electrophoresis showing double enzyme digests of transformants selected from four PCR cloning experiments and four subcloning experiments. These demonstrate 100% recombination efficiency, based on the random selection of between 3 to 5 clones per ligation/transformation experiment. Images **a**) & **b**) show PCR-generated p1108 clones digested with *Bam*HI and *Eco*RI. **a**) Lanes 2-5 = p1108-85A, lanes 6-8 = p1108-ESAT-6. **b**) Lanes 2-4 = p1108-tPA-85C, lanes 5-7 = p1108-tPA-ESAT-6. Images **c**) & **d**) show pWR510 subclones digested with *Nhe*I and *Eco*RI. **c**) Lanes 2-6 = pWR510-85A, lanes 9-13 = pWR510-tPA-85A, lanes 7 and 14 = p1108-tPA-85A (positive control). **d**) Lanes 2-6 pWR510-ESAT-6, lanes 9-13 = pWR510-tPA-ESAT-6, lanes 7 and 14 = p1108-tPA-ESAT-6 (positive control). MW=100 bp molecular weight marker.

purification step and Microcon buffer exchange and the stock concentration was adjusted to 1 µg/µl. Figure 4.11 shows an example of *Bam*HI/*Eco*RI digested Midipreps.

4.2.4 Sequencing PCR clones

Following construction, all PCR clones were sequenced across the cloning site to confirm identity of the insert (carried out by Dr J. McFadden's laboratory, University of Surrey prior to sequencer being available locally). These data also confirmed the correct cloning and sequence of tPA in the two tPA vector derivatives (four forward runs for p1108-tPA and three forward runs for pWR510-tPA) (sequence data not shown). Subsequently, a selection of PCR clones were sequenced in more detail using both forward and reverse sequencing primers at LSHTM. These included p1108-tPA constructs for all four genes and pWR510-tPA-85C. No errors were identified in any of the constructs.

4.2.5 Generation of rVV

rVV construction was performed as described in Section 2.15. Eighteen rVV were made using all the plasmids listed in Table 2.1 except #12, #15, #17 and #20. All the rVV were constructed simultaneously. Thus, a complete set of eight rVV, both with and without the signal sequence, was prepared for genes under the control of the p7.5 promoter. Four functional rVV were prepared for use in the vaccinia/T7 hybrid system (85B and 85C constructs), plus two non-functional ESAT-6 constructs.

4.2.6 Verification of rVV construction using PCR

4.2.6.1 Purified rVV genome required for PCR

As described in the literature (Pasamontes et al. 1991), several attempts were made to verify rVV construction using PCR templates taken directly from the rVV-infected cell lysates. A variety of primers were tried including: 23/24 (pWR510), 25/26 (within the tPA region) and 28/29 (*tk* homology), but no signals were identified (see Table 2.4 for primer details). Therefore, genomic preparations were prepared for each of the rVV as described in Section 2.16.1.1. Using these purified templates, PCR amplification was then demonstrable.

4.2.6.2 Assessment of flanking and tPA primers for verifying rVV recombination

Figure 4.12 shows the result of testing a variety of primer pair combinations for their ability to verify vaccinia recombination. Details of the primers and their predicted product size is given in Table 2.4. Products of PCR reactions were run in pairs, the rVV genomic

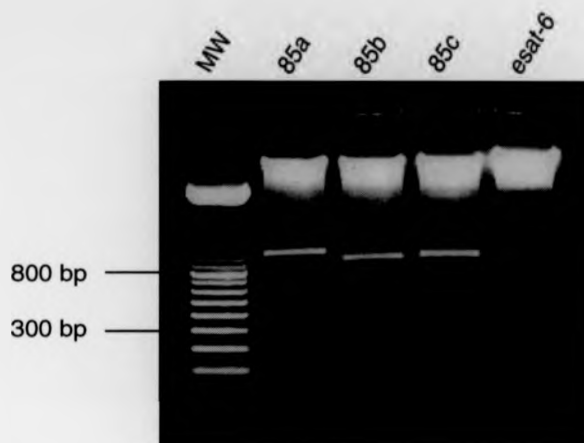


Figure 4.11 Double digest of purified 'Midiprep' p1108-tPA-constructs

Following identification of plasmid recombinants, a single clone was selected for each of the generated gene/vector combinations. Medium scale plasmid preparations (Midiprep, Promega) were performed and the products were subjected to phenol/chloroform and spin column purification. These highly purified plasmid preparations were used for sequencing and vaccinia recombination. The agarose gel shows an example of four constructs, p1108-tPA-85A, -85B, -85C and -ESAT-6, each digested with *Bam*HI and *Eco*RI. MW=100 bp molecular weight marker.

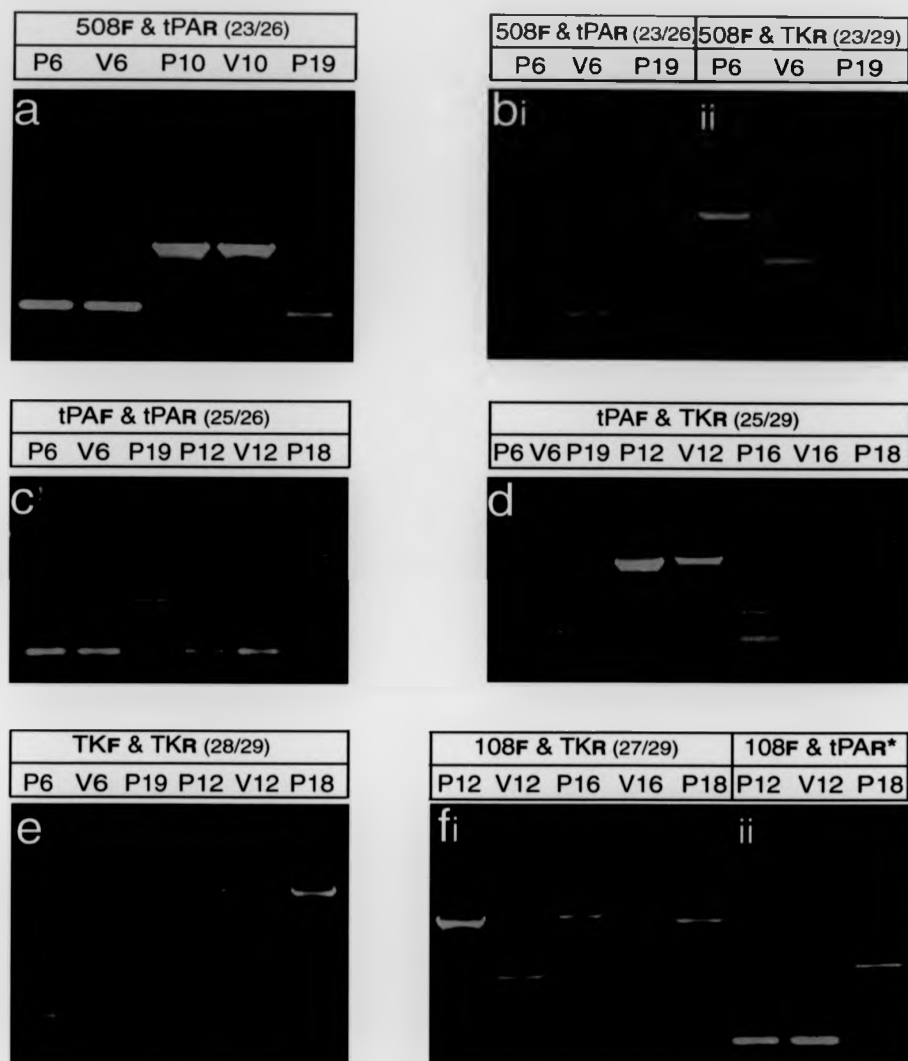


Figure 4.12 Identification of primers suitable for verification of rVV

PCR was used to verify successful integration of the gene inserts into wild-type vaccinia. Purified genomic DNA, derived from rVV-infected cell lysates, was used as the PCR template and a variety of primer pairs were assessed for their ability to amplify across the cloning site (applicable for all constructs) and also from within the tPA region (constructs with tPA). The genomic preparation of an rVV was denoted as V plus digit and the corresponding plasmid was used as a positive control (P plus digit)(see table 4.1 for details). Primer abbreviations are coded by the names of the homologous region: 510=pWR510, 108=p1108, tPA=tPA region and TK=thymidine kinase. F & R=forward and reverse primers. The primer unique identifier is given in brackets (see table 2.4). * = 27/26

preparation (coded V + unique identifier) and the corresponding insertion plasmid (P + unique identifier), acting as a positive control e.g. V10=rVVT7-tPA-85C and P10=pWR510-tPA-85C (see Tables 2.4 and 4.1). As an additional control, an appropriate non-recombinant plasmid (p1108 or pWR510) was also included for each of the primer pairs tested.

Figure 4.12a shows discrete strong signals for both plasmid and rVV template reactions. This primer pair, 23/24, flanks the cloning site of pWR510. The tPA forward and reverse primer pair, 25/26, was equally effective (Figure 4.12c). However, this pair also resulted in mispriming with the control plasmids lacking tPA, producing a band of ~1.5 kb (the product size is demonstrated in Figure 4.13a(ii) lanes 10 and 11). The same false positive result was also seen in other primer pair combinations which used the tPA antisense primer (Figure 4.12 b and e). This would suggest binding of this primer to the other strand within the *tKL* region, as the same size product seen for both pWR510 and p1108.

Other primer pair combinations showed variable efficacy. Of interest, combinations which included the TKR antisense primer would amplify a product from plasmid but not rVV (see Figure 4.12b(ii), e(i) and f).

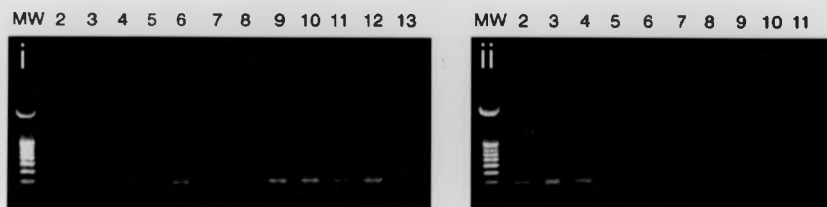
4.2.6.3 PCR verification of rVV-tPA and rVV-T7 constructs

Following the identification of the best primer pairs for verifying recombination, all rVV were tested for tPA insertion and a pWR510 background. Evidence for successful recombination (for the PCR positive constructs) was supported by a signal of the correct molecular weight in all the rVV-tPA constructs and none of the rVV lacking this region (Figure 4.13a). Furthermore, using the pWR510 primer pair, 23/24, PCR signals of the predicted molecular weight were seen for all the rVVT7 constructs and their corresponding plasmids and none of the rVVp7.5 constructs (Figure 4.13b). This confirmed insertion of a fragment of the correct molecular weight in all the T7 constructs.

4.2.6.4 Use of cloning primers to verify rVV

In addition to the tPA and flanking primer pairs described above, the cloning primers were also used to verify vaccinia recombination. These data are shown in Figure 4.14, where evidence for recombination exists for most, but not all of the constructs. Signals were absent for rVVp7.5-85B and rVVp7.5-ESAT-6 and their plasmid controls (Figure 4.14a, lanes

a



b

gene	<i>Ag85b</i>				<i>Ag85c</i>				<i>esat-6</i>				tPA	
tPA	-	-	+	+	-	-	+	+	-	-	+	+	+	+
virus or plasmid	v	p	v	p	v	p	v	p	v	p	v	p	v	p

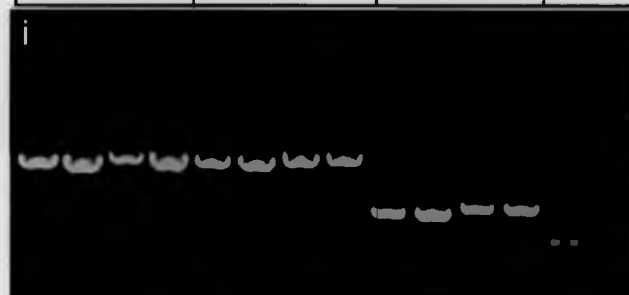


Figure 4.13 PCR verification of rVV using tPA internal primers and pWR510 flanking primers

a) All the rVV were assessed for the integration of tPA, as indicated by a PCR signal using the primer pair, 25/26. A signal of the correct size was obtained in: **i** lanes 6, 9-13; and, **ii** lanes 2-4, 8 and 9. These signals corresponded with the predicted pattern for all the rVV-tPA constructs and none of the rVV lacking tPA. **b)** PCR using the primer pair 23/24 which flanks the pWR510 coding region. This demonstrated amplification of: **i** all the pWR510-derived constructs and, **ii** none of the p1108-derived constructs (lanes 2-11). In addition, no signal was achieved if the PCR template was non-purified cell lysate (**ii** lane 12, rVVT7-tPA-85C) rather than the genomic preparation from the same rVV (**i** lane 7). Lane 13 shows the positive control, where pWR510 was used as the PCR template. MW=100 bp molecular weight marker.

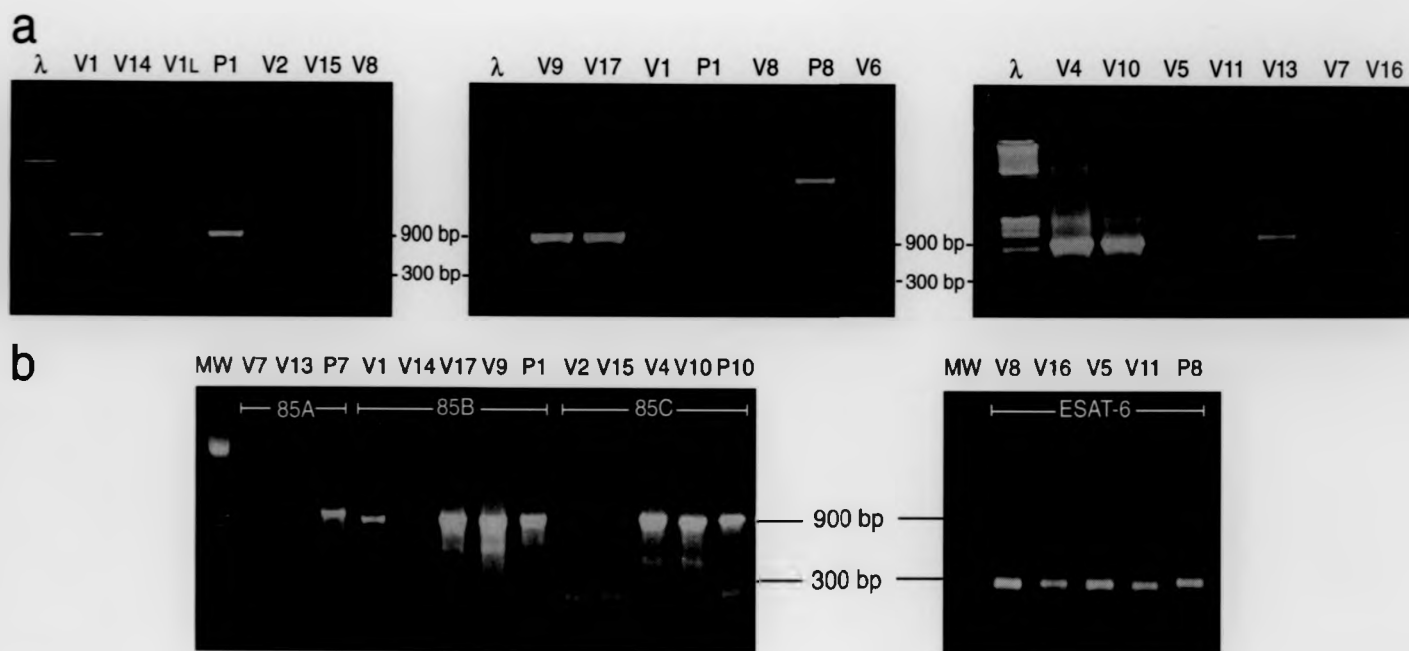


Figure 4.14 PCR verification of rVV using cloning primers

In order to verify integration of the gene inserts, PCR was performed using the same cloning primers used to generate the inserts. Purified genomic preparations of rVV-infected cell lysates were used as template, except in one case (top left hand gel, lane V1L) where the unpurified cell lysate was used. Groups of gels represent two replicate experiments, **a**) & **b**). Signals of the predicted size were obtained for all constructs, in at least one of the two experiments, except for: V14 (p1108-derived rVV-tPA-85B) and V2 (p1108-derived rVV-85C). Table 4.1 gives details of the construct number code. The type of construct used as PCR templates are indicated as: V = genomic DNA from rVV-infected cell lysates and P = plasmid template. MW=100 bp molecular weight marker.

12-15) and was only weakly discernable for rVVp7.5-85C (lane 6). Figure 4.14b shows a repeat PCR for all the rVV grouped according to gene and each with a plasmid control for size comparison. A signal of the correct size was seen in all cases except rVVp7.5-tPA-85B (lane 6) and rVVp7.5-85C (lane 10).

4.2.6.5 Identification of flanking primers for p1108 and rVVp7.5 constructs

Initially, no sequencing data was available downstream of the p1108 MCS (Dr Mike Mackett, personal communication). Following construction of the p1108-gene recombinants and sequencing the 3' end of the sense strand, a primer was selected (PR10840R, Table 2.1). In combination with PR10827F, this primer pair readily amplified all inserts in both p1108-derived rVV and the corresponding plasmids (Figure 4.15). Thus, these data taken together provide evidence for successful gene integration into vaccinia in all cases.

4.3 DISCUSSION

Twenty plasmids were prepared using p1108 and pWR510 vectors. A second generation of eighteen rVV were constructed. The desired goals of *Ag85* and *esat-6* gene integration, tPA signal sequence insertion and gene integration under the T7 promoter were achieved in all possible combinations, except for the rVVT7-85A and rVVT7-ESAT-6 constructs. However, a variety of methodological issues arose during the different stages of development. These are discussed by summarising the achievements, pitfalls and mistakes experienced.

4.3.1 Achievements

4.3.1.1 Cloning primers

The computer programme-generated primer predictions produced strong signals in all PCR reactions enabling the use of *Pfu* polymerase in all but one case, 85a into p1108, for which *Taq* polymerase was used. The inclusion of restriction sites and manipulation of the start or stop codon was successful. The only insert which could not be cloned was 85a into pWR510. The reasons for this are unclear. Where PCR clones were not obtained (four pWR510 constructs), subcloning from p1108-tPA-85A and -ESAT-6 was possible due to the presence of both *Bam*HI and *Nhe*I sites in the tPA MCS.

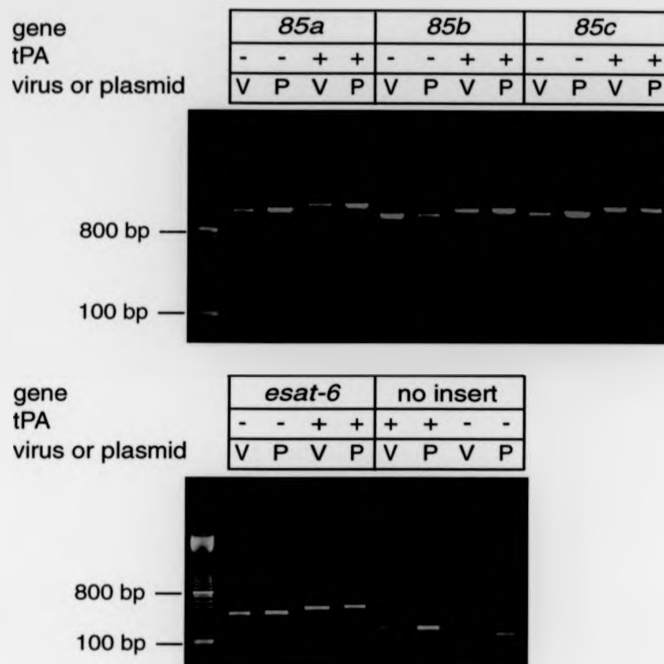


Figure 4.15 PCR verification of p1108-derived rVV

A primer pair flanking the cloning site was used to verify vaccinia recombination. The PCR templates were: the genomic preparation of a p1108-derived rVV-infected TK-143 cells (V) paired next to the corresponding p1108 plasmid recombinant (P), with each plasmid acting as a positive control for the rVV. tPA- = no signal sequence, tPA+ = signal sequence present. Left hand lanes=100 bp molecular weight marker.

4.3.1.2 *p1108-tPA and pWR510-tPA*

Construction of the tPA insertion plasmids was achieved and sequence fidelity was confirmed. This provided useful generic constructs with a MCS permitting relatively simple sticky or blunt-ended cloning. This was not only useful for this study, but would also be of benefit for those wanting to create rVV-tPA fusions.

4.3.1.3 *High cloning efficiency and membrane filtration*

All inserts were readily cloned into their respective insertion plasmids with very high recombination efficiencies, reaching 100% for the final 8 ligations. In part, this was facilitated by the cloning strategy which used membrane filtration with reduction in the salt content and the unwanted sticky-ended fragments following digestion. The high recombination efficiency permitted omission of PCR screening of bacterial colonies and selection of as little as three transformants for each ligation reaction. For the subcloning experiment, membrane filtration avoided the use of a phenol/chloroform purification step of cut insert following electroelution from the cut gel slice. Instead, the ~500 µl volume was washed and concentrated (a 30m procedure). In addition, the cut vector was processed using the Micropure EZ insert (enzyme removal) and membrane filtration. From four ligations, all 20 clones selected contained a correct insert. However, no direct comparison of efficiency between phenol/chloroform purification and the Microcon system was made and the high cloning efficiency may have been due to other factors. Importantly, the Microcon system avoids some phenol/chloroform steps, thus improving safety and reducing processing time.

4.3.1.4 *Construction of rVV and PCR verification*

Construction of 18 rVV was achieved. Primer design and assessment of PCR verification produced clear results for primer pairs flanking the cloning sites of both pWR510-derived and p1108-derived rVV, as well as for tPA-containing rVV. Additional, but incomplete evidence was provided using the cloning primers. Without sequencing data, none of these PCR data provide absolute proof of correct gene integration. Flanking primers merely indicate that a correctly sized fragment has been cloned. Cloning primers could potentially 'verify' any PCR insert generated by the same primer pair e.g. the product of mispriming from the original BlueScribe vector carrying the gene. However, all plasmid clones were sequenced prior to vaccinia recombination, making this very unlikely. An alternative approach would have been to use a primer pair amplifying an internal region within each

gene, perhaps selected to distinguish the different *Ag85* genes. In addition, a combination of flanking primers and internal primers might have permitted successful nested PCR and obviated the need for preparation of rVV DNA, a purification step required in this study but not by others (Pasamontes et al. 1991).

4.3.2 Pitfalls and mistakes

4.3.2.1 *XbaI in the forward primer*

XbaI was chosen as the selected site for the upper primer in several cases (01, 05 and 09). The programme gave higher scores for these choices as compared with the equivalent primer with a *NheI* site. Whilst this produced a compatible sticky end for cloning into pWR510, the *NheI* site would then have been lost, making subsequent recombination more difficult to verify and losing the ability to sub-clone. In the final event, none of these primers were used for plasmid construction.

4.3.2.2 *Errors in primer design*

Two errors were introduced into primers 06 and 17 at the time of their design. This was discovered early on in the case of the *85b* PCR product and the mistake rectified. However, the error in primer 17 was only identified following rVVT7-ESAT-6 construction (during the alignment of sequence runs). In retrospect, a computer-generated plasmid sequence and protein translation would have identified this problem prior to primer purchase.

4.3.2.3 *A need for early sequencing of clones*

Two PCR clones used *Taq* polymerase-generated inserts (p1108-85A constructs). Given the relatively lower fidelity of this enzyme, these clones should have been fully sequenced as soon as possible after construction. Ideally, the same is also true for the *Pfu*-generated PCR products, although the risk of misincorporation is much less. This would identify sequence or cloning errors and permit early correction. In addition, rVV which do not appear to express could be further explored without the worry of a potential PCR misincorporation error. From a practical perspective at the time of the cloning, no automated sequencer was available in our institute. All plasmids were sent away, but because of the relatively high expense, only sequencing across the cloning site was performed. Manual sequencing of so many clones would have been very time consuming. At a later date when an automated sequencer was available on site, eight plasmids were sequenced in both directions with no

evidence of misincorporation (the two *Taq*-generated clones, all the four p1108-tPA clones and the two tPA-85C clones).

4.3.2.4 Subcloning rather than PCR cloning

Whilst *Pfu* polymerase generates inserts with a relatively low chance of misincorporation, this chance is still significantly greater than subcloning a fully sequenced insert. In retrospect, a more efficient cloning strategy would have been construction of all four p1108-tPA-gene constructs with full sequencing of these, followed by subcloning into p1108, pWR510 and pWR510-tPA. However, construction of p1108-tPA took a relatively long time and the other reagents were fully prepared and ready for use (cut vectors and cut PCR inserts). Therefore, the construction of PCR clones was chosen. However, despite the lack of availability of p1108-tPA early in the cloning experiments, subcloning could still have been performed from p1108 to p1108-tPA and likewise, for pWR510 to pWR510-tPA, thus limiting the number of clones requiring sequence verification.

4.4 CONCLUSION

Twenty insertion plasmids were constructed from vectors p1108 and pWR510, using both PCR and subcloning. Two of these, p1108-tPA and pWR510-tPA represent generic vaccinia insertion plasmids for rVV expression of genes, fused downstream from the eukaryotic tPA signal sequence and under the control of either p7.5 or T7 promoters. Sixteen of the plasmids plus the two vectors were used to construct eighteen rVV. The remaining four pWR510 plasmids (*85a* and *esat-6* with and without tPA) were prepared for *in vitro* transcription/translation experiments. All PCR clones were sequenced across the cloning site and eight were sequenced in both directions. Successful integration of tPA and MTB genes into vaccinia was confirmed using a variety of flanking, tPA and cloning primers.

5 Antigen 85 and ESAT-6 protein expression

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5.1 INTRODUCTION

The construction of the rVV, as described in Chapter 4, was apparently successful, judged at a DNA level. However, it was important to assess the expression of the recombinant antigens before the rVV could be used in immunological assays. This chapter describes the results of two methods used to assess protein expression from both rVV and plasmid constructs. For rVV expression, virally-infected cell lysates were subjected to immunoblotting using both dot and Western blotting techniques. *In vitro* transcription/translation was employed using the pWR510 constructs containing MTB genes under the control of the T7 promoter. This cell free system permitted further protein analysis using constructs with different mycobacterial genes, both with and without the signal sequence. In addition, protein expression for rVV-ESAT-6 constructs was demonstrated by collaborators using a newly developed capture ELISA. In chapter 6, the results of immunofluorescence are shown in order to demonstrate recombinant protein expression in rVV-infected cultured human macrophages which were used as CTL target cells.

5.1.1 Immunoblotting

Immunoblotting represents one of three common means to detect recombinant protein (Mackett, 1995). Whilst immunofluorescence staining of cells infected with rVV represents a straightforward and fast method, it would not have permitted assessment of molecular weight differences or multiple bands resulting from post-translational modification. Size resolution and a high degree of sensitivity are possible with immunoprecipitation. However, no metabolically-labelled culture filtrate protein positive control was available.

In these experiments, the Amersham ECL chemiluminescent protocol was used in all cases, as described in Section 2.16.3. A preliminary experiment compared diaminobenzidine (DAB, Sigma) staining and the chemiluminescence protocol; each utilised the same secondary antibody conjugated to horse radish peroxidase. The DAB readout was less sensitive (data not shown) and was not employed in further experiments.

5.1.2 *In vitro* coupled T7 transcription/translation

The bacteriophage T7 promoter is particularly powerful, resulting in very high levels of expression in many systems (Moss et al. 1990). Section 1.6.7 discusses this in more detail with particular reference to two systems: the vaccinia/T7 hybrid system and *in vitro* cell free transcription/translation. This study utilised both methods in order to address two questions:

Was the failure to observe rVV-ESAT-6 expression under p7.5 was due to low rather than absent protein expression? and; What was the effect of a eukaryotic signal sequence in a cell free system? The construction of rVV encoding MTB secreted proteins under the T7 promoter (designated rVVpT7 constructs) would help assess absent versus low-level expression of protein. *In vitro* transcription/translation using the pWR510 constructs would allow dissection of protein properties by comparing the impact of the signal sequence using the system both with and without microsomal membranes. Additional fractionation of the microsome reaction products into membrane-associated protein and protein in the supernatant would provide further evidence for post-translational modification. Thus, the pWR510 constructs were able to serve a dual function, as insertion plasmids for homologous recombination, and as templates for cell free protein expression.

5.2 RESULTS

5.2.1 Immunoblotting of rVV-Ag proteins

In order to identify proteins of the Ag85 complex, dot blot and Western blot experiments were performed using two mAbs, TD-17 and TD-32 (Table 2.5). Results from the latter mAb produced an identical signal pattern but of lower intensity. Most of these data are not shown. Later in the project a further polyclonal antibody became available and was used to probe the same virally-infected cell lysates. For the purpose of comparison, blots using both the mAb, TD-17 and the polyclonal antibody, K92, are shown in the same figure.

5.2.1.1 Dot blotting of rVV-Ag85 infected cell lysates

Twofold dilutions of rVV-Ag85-infected cell lysates were directly dotted onto a nitrocellulose membrane, dried and probed with mAb TD-17 using the protocol described in Sections 2.16.3.6-7. MTB culture filtrate (CF) was used as a positive control and rVV-tPA alone was used as a negative control. For comparison, a similar experiment was performed for the 85B constructs using the polyclonal antibody, K92. The results are shown in Figure 5.1. As well as the positive control, specific signals were detected for all 85A and 85B constructs, but not for 85C. Furthermore, in all cases where a specific signal was detected, the intensity was greater for tPA fusion protein than the corresponding non-tPA recombinant protein. For mAb TD-17 and 85A, the addition of tPA accounted for approximately an eightfold increase. With the same antibody, for 85B, the signal was approximately 2-4 fold greater. However, with the polyclonal antibody, the tPA-85B signal was approximately 8-16 fold greater than 85B alone.

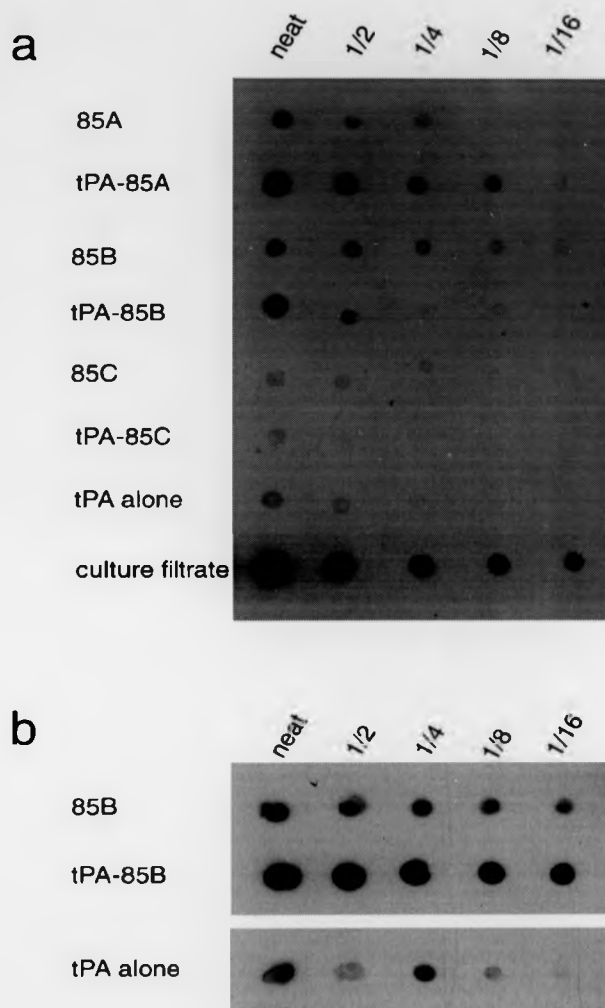


Figure 5.1 Dot blots of rVV-infected cell lysates probed for Ag85
rVV-Ag85 infected cell lysates and *M. tuberculosis* culture filtrate (CF) were dotted in 1 μ l aliquots onto nitrocellulose membrane, in twofold serial dilutions and probed using: **a)** mAb TD-17; or **b)** rabbit polyclonal anti-MPT59 (Ag85) antiserum. The signal was created using peroxidase-conjugated secondary antibody and chemiluminescence (ECL, Amersham).

5.2.1.2 *Western blotting using the mAb TD-17*

Using the same protein samples as used for dot blotting, Western blotting was performed with 15 µl of protein loaded per well. The initial data, using the mAb, is demonstrated in Figure 5.2a(i). This shows strong single bands for both rVV-85A and tPA-85A. Three bands of lower intensity are seen in the rVV-tPA-85B lane and a very strong signal from the CF positive control. The 85A band is in keeping with the predicted 32 kDa size. Only an approximate comparison can be made with the positive control in this blot (the signal is too strong for clear comparison), but equivalent molecular size is clearer in a similar experiment shown in Figure 5.2b(i). The band seen in rVV-tPA-85A is of a larger size than would be predicted, given that it encodes only a further 21 residues. The same is certainly true for tPA-85B which, in native mycobacterial form, is only 30 kDa in size (as the mature protein found in culture filtrate).

An underexposed Western blot containing the same protein was probed with the mAb, TD-32. Whilst a lower signal was apparent, the results illustrated in Figure 5.2a(ii) demonstrate two discrete bands in the CF control, consistent with the two dominant 85A and 85B proteins in mycobacterial CF (32 kDa and 30 kDa respectively).

A repeat Western blot was performed using the same protein concentration in each lane (the samples were previously equilibrated for concentration). This demonstrated the same banding pattern as seen in Figure 5.2a(i) (data not shown). In addition, both a short and long exposure were performed (Figure 5.2b). These demonstrated that all the 85A and 85B recombinants produced a signal indicating expression. This included rVV-85B (without tPA) not seen in the previous Western blot. Even in the over-exposed autoradiograph, there was still no evidence for 85C expression.

5.2.1.3 *Western blotting using the polyclonal antibody K92*

When the polyclonal antibody became available, the Western blotting experiment was repeated using the same protein samples and K92 antibody. The result is shown in Figure 5.3a. For comparison, a detail from Figure 5.2a (the same Western blot probed with mAb TD-17) is given below (Figure 5.3b). Using the polyclonal sera, signals from the rVV-85B constructs were now similar to the rVV-85A constructs. As with the mAb probe, three higher molecular weight bands were noted for rVV-tPA-85B. The presence of multiple bands of a higher molecular weight than expected suggested post-translational modification

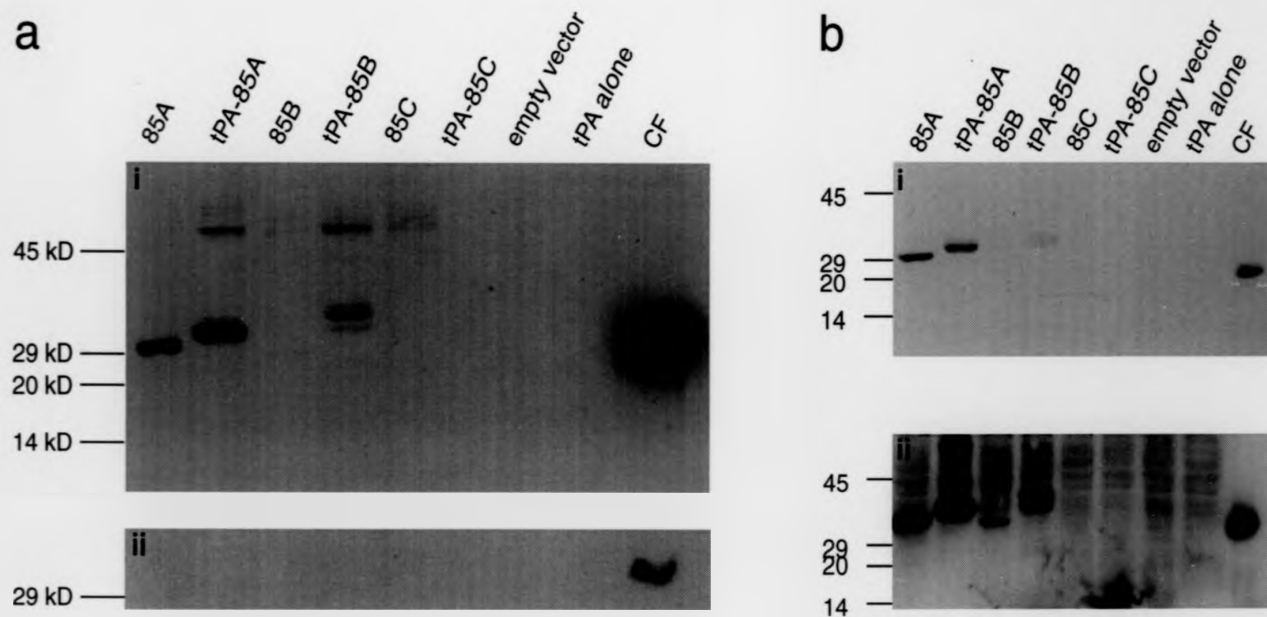


Figure 5.2 Western blots of rVV-infected cell lysates probed for Ag85 using two monoclonal antibodies

a)i 10 μ l of cell lysate per lane was run on SDS-PAGE, blotted onto nitrocellulose membrane, probed with mAb TD-17 and the signal detected using chemiluminescence. **ii** This is a detail showing the only signal present on an identical blot to **a)i** except that in this case it was probed with mAb TD-32. Note the double band in the CF lane. **b)i & ii** These images show the same experiment as **a)i**, but include different autoradiographic exposures of the same blot. **i** A brief 45s exposure and **ii** a prolonged 15m exposure.

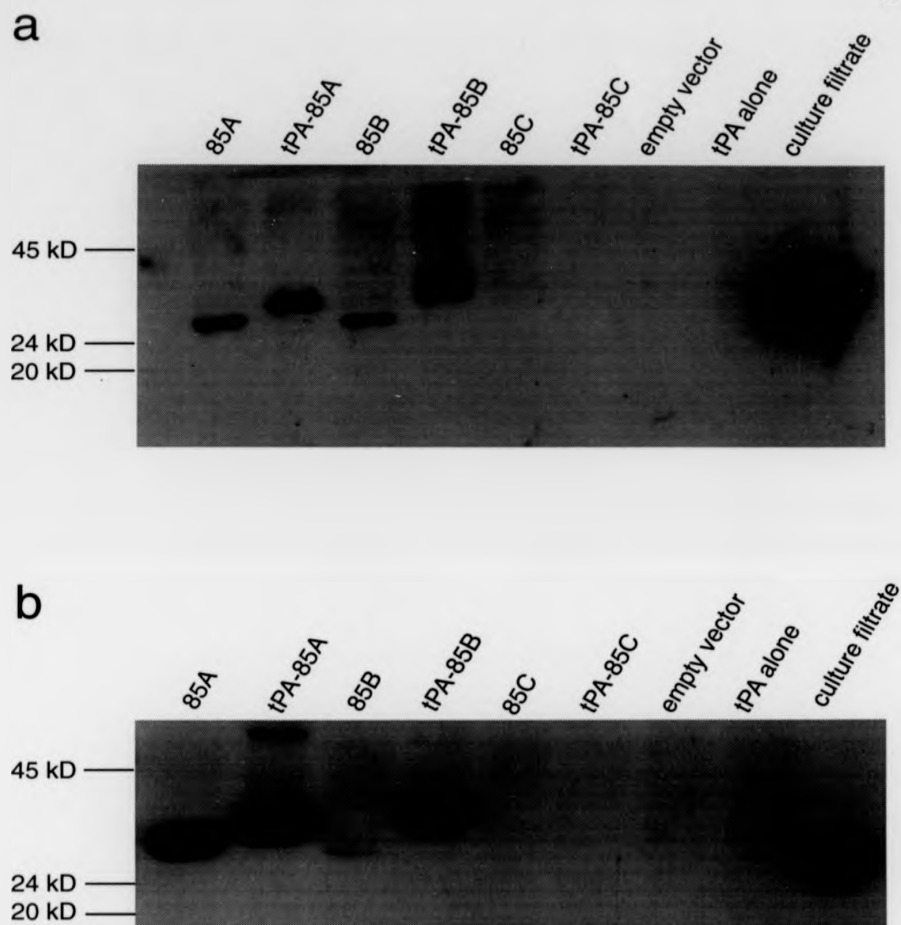


Figure 5.3 Western blots of rVV-infected cell lysates probed for Ag85 with polyclonal and monoclonal antibodies

rVV-Ag85 infected cell lysates (10 μ l loaded per lane; lanes 1-8) and *M. tuberculosis* culture filtrate (1 μ l loaded; lane 9) were run on SDS-PAGE, transferred to nitrocellulose membranes and probed using: **a)** rabbit polyclonal antiserum - K92; or **b)** mAb TD-17. The signal was created using peroxidase-conjugated secondary antibodies and chemiluminescence (ECL, Amersham).

of the recombinant protein. Further evidence for this is provided below. As for the mAb Western blot, no signal was seen for the 85C constructs.

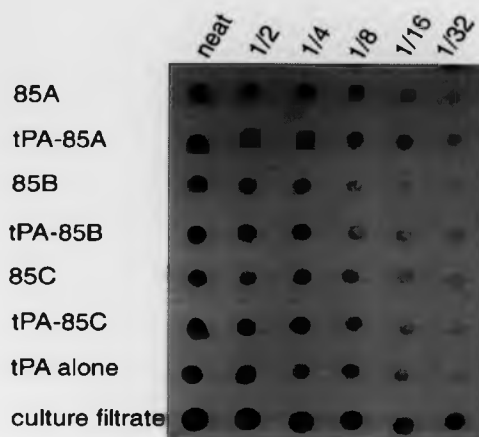
5.2.1.4 *Western blotting using immune sera from Ag85 DNA vaccinated mice*

In order to identify possible 85C expression, several mouse polyclonal sera were used in Western blotting studies. These were derived from mice vaccinated with a variety of Ag85 DNA constructs including 85C. Previous work by K. Huygen had demonstrated that the 85C construct was immunogenic along with the other DNA constructs (personal communication) (Huygen et al. 1996). Attempts at dot blotting with these sera was unsuccessful, the background being too high to distinguish a specific signal except for the CF positive control (Figure 5.4a). However, a Western blot probed with serum from a mouse vaccinated with 85A DNA showed a strong signal for 85A, tPA-85A and CF (Figure 5.4b). Serum from a mouse vaccinated with 85B DNA produced a weak signal of the correct size for the CF control only (Figure 5.5a). For anti-85C, amongst non-specific binding, there were signals consistent with identification of 85A, tPA-85A, 85B, tPA-85B and CF, but not 85C (Figure 5.5b). A further serum sample came from a mouse vaccinated with all three DNA constructs (labelled anti-85A-C). Probing with this serum produced a pattern similar to the anti-85C blot (Figure 5.5c). It would be tempting to believe that the signal in the 85C lane, of about the correct size, might suggest the presence of this protein. However, similar signals were also evident in the negative controls.

5.2.1.5 *Evidence for glycosylation of rVV-tPA-85B*

The previous Western blots identified three discrete bands for rVV-tPA-85B suggesting three possible glycoforms of the same protein. In order to confirm that these were glycoforms, N-glycosidase digestion of cell lysates were performed for this construct and 85B without tPA (Section 2.16.3.8). Figure 5.6 shows a Western blot of digested and undigested cell lysates, probed with the polyclonal antibody, K92. Lanes 1 and 2 show that the enzyme had no effect on the non-tPA construct. However, for tPA-85B, all three bands were lost following N-glycosidase digestion and were replaced with two further bands. The lower molecular weight band could represent non-glycosylated tPA-85B and the higher molecular weight band, a glycoform not digested with this enzyme

a



b

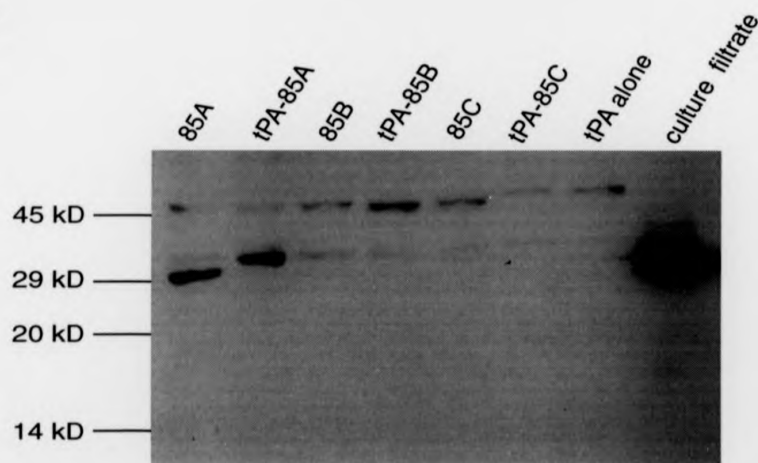


Figure 5.4 Immunoblots probed with serum from a mouse immunised with an Ag85A DNA vaccine

a) A dot blot showing 1 μ l aliquots of rVV-infected cell lysates tested neat and in a series of twofold dilutions and probed with serum from a mouse previously vaccinated with a DNA vaccine encoding the tPA-Ag85A sequence. **b)** A Western blot using the same cell lysates (10 μ l loaded per lane) and antibody probe.

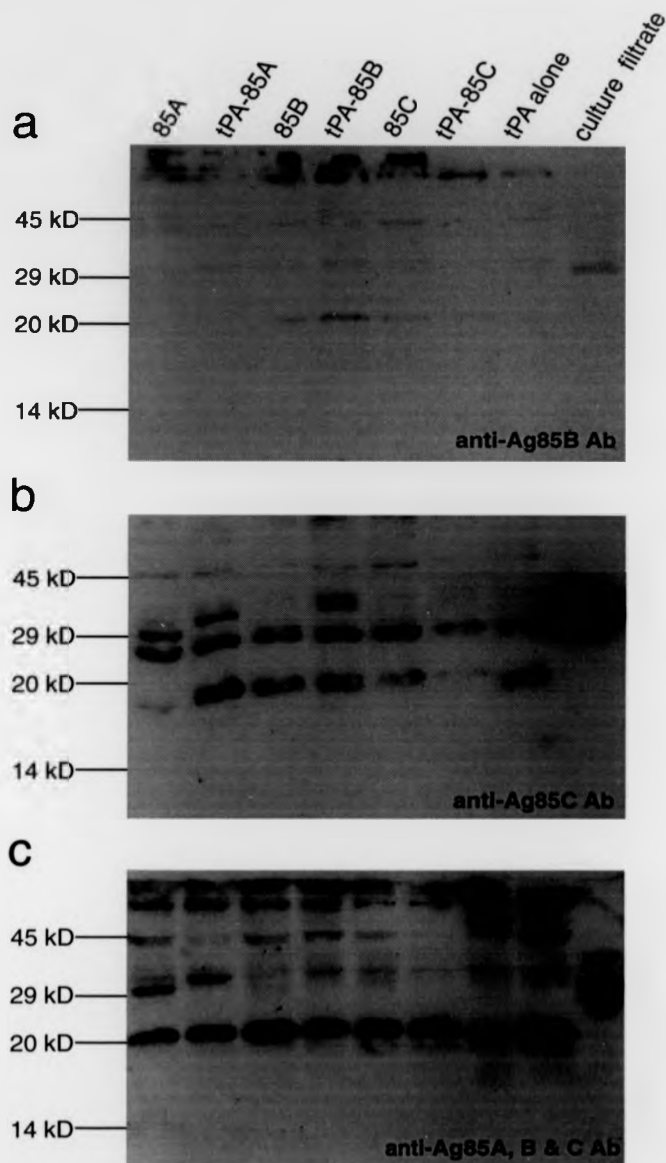


Figure 5.5 Western blots probed with serum samples from mice immunised with one or multiple Ag85 DNA vaccines

rVV-Ag85 cell lysates were subjected to Western blot analysis using three different serum samples from individual mice immunised with either: **a)** a DNA vaccine encoding the tPA-85B sequence (top image); **b)** a DNA vaccine encoding the tPA-85C sequence (middle image); and **c)** three DNA vaccines encoding the tPA-85A, tPA-85B and tPA-85C sequences respectively (lower image).

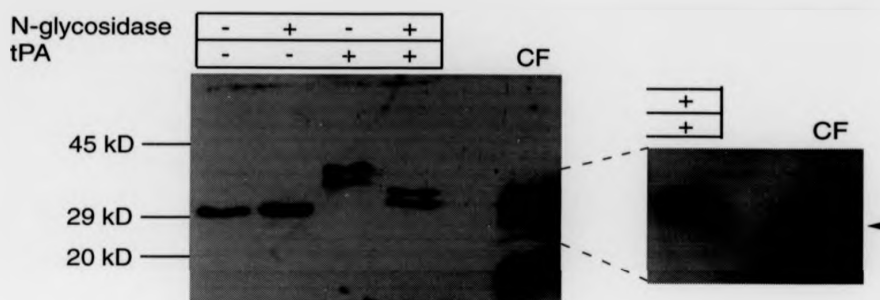


Figure 5.6 Western blot of N-glycosidase digested rVV-Ag85B lysates

Cell lysates, previously infected with rVV-Ag85B (lanes 1 and 2) or rVV-tPA-Ag85B (lanes 3 and 4), were digested (lanes 2 and 4) or mock digested (lanes 1 and 3) with N-glycosidase and then probed using a rabbit polyclonal anti-Ag85 antisera (K92). The detail shows an enlarged version of the CF lane from a replicate experiment where the signal for the Ag85 complex is more distinct (indicated by the arrow head). CF=*M. tuberculosis* culture filtrate (positive control).

5.2.1.6 *85B expression using the T7 promoter and comparison with the p7.5 promoter constructs*

Of the four rVVpT7 constructs encoding *85b* and *85c* both with and without tPA, immunoblot signals were identified for the 85B constructs only. As for the rVVp7.5 constructs, the addition of tPA enhanced expression, as shown using dot blotting (Figure 5.7a). In addition, a Western blot also showed a similar banding pattern to the rVVp7.5-generated protein (Figure 5.7b). Despite replicate experiments and careful attention to quantifying the viral titres, there was no evidence for enhanced expression using the T7 vaccinia hybrid system (Figure 5.7a).

5.2.1.7 *Second generation rVV-ESAT-6 constructs*

Cell lysates derived from p1108-derived rVV-ESAT-6 and rVV-tPA-ESAT-6 constructs were probed with the mAb HYB 76-8 in both dot and Western blot experiments. SDS-PAGE gels were run using reducing and non-reducing conditions. An alternative HYB 76 mAb clone, HYB 76-13, was also used for immunoblotting. Despite the development of the tPA-ESAT-6 fusion, immunoblotting failed to detect a positive signal. The following year, Prof. M. Harboe's group in the University of Oslo, Norway, developed a capture ELISA, utilising the original mAb, HYB 76-8, in the first layer, the test reagent in the second layer and a polyclonal anti-peptide antibody in the third layer (see below for description, Section 5.3.2.3). They assessed rVV-ESAT-6 cell lysates from this study using their capture ELISA and demonstrated high level protein expression for rVV-tPA-ESAT-6 (Harboe et al. 1997). Protein expression was also seen for rVV-ESAT-6, but at a much lower level. These results are summarised in Figure 5.8, which was kindly provided by Prof. M. Harboe.

5.2.2 *In vitro coupled T7 transcription/translation*

5.2.2.1 *Transcription/translation without microsomes*

Following subcloning of genes *85a* and *esat-6* from the appropriate p1108-tPA plasmids, a full set of pWR510 constructs were available for *in vitro* transcription/translation experiments (Section 2.11.3.3). Half a microgram of purified plasmid was added per reaction tube along with the other appropriate reagents, minus microsomes. Metabolic labelling was performed using ^{35}S methionine. Further details of the methodology are described in Section 2.17. Figure 5.9 shows results from three separate experiments represented in the autoradiographs labelled 'a', 'b' and 'c'. Figure 5.9a shows metabolically-

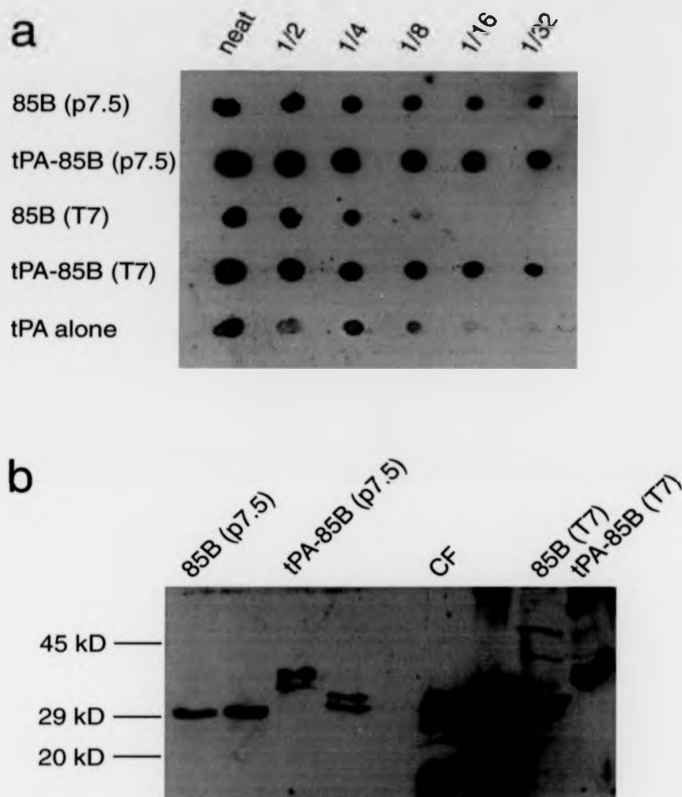


Figure 5.7 Immunoblots of rVV-85B-infected cell lysates - p7.5 versus T7 promoter
a) rVV-85B infected cell lysates were prepared using constructs utilising the T7 promoter (pWR510-derived) and the p7.5 promoter (p1108-derived). Protein samples were dotted using 1 μ l aliquots, both neat and in a series of twofold dilutions. The membrane was then probed with a rabbit polyclonal antibody (K92). **b)** The Western blot shows rVV-85B infected cell lysates (p7.5 promoter constructs in lanes 1 and 3, T7 promoter constructs in the two far right lanes) also probed with K92. A similar banding pattern was produced irrespective of the promoter. Different protein concentrations were used, therefore no quantitative comparison can be made. Unlabelled lanes represent data from a separate experiment. CF=MTB culture filtrate.

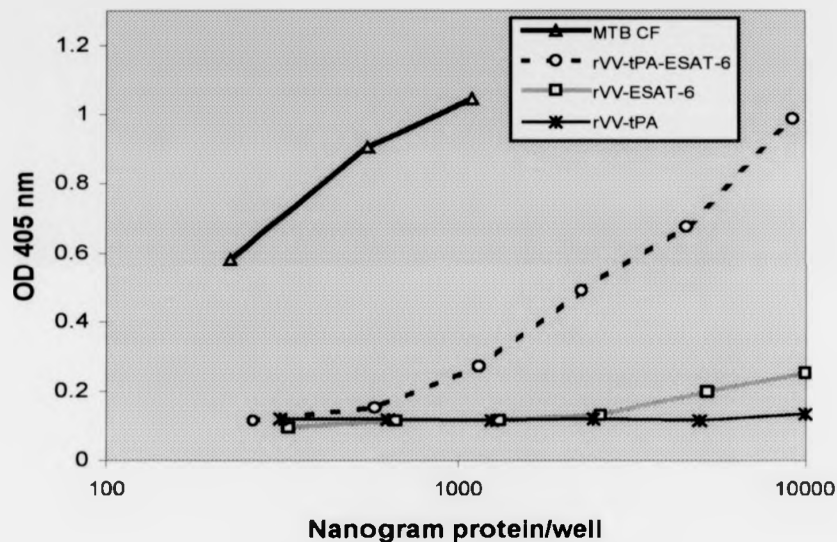


Figure 5.8 Capture ELISA for ESAT-6

Protein was obtained from rVV-infected cell lysates (this study) and MTB CF (ST-CF from the Statens Seruminstitut, Copenhagen). In Oslo, protein quantification was performed using the same technique for all samples (BCA protein assay reagent, Pierce Chemical Comp., Rockford, IL, US). Serial dilutions of protein were then assayed for ESAT-6 using a capture ELISA and the OD₄₀₅ measured (Harboe et al. 1997).

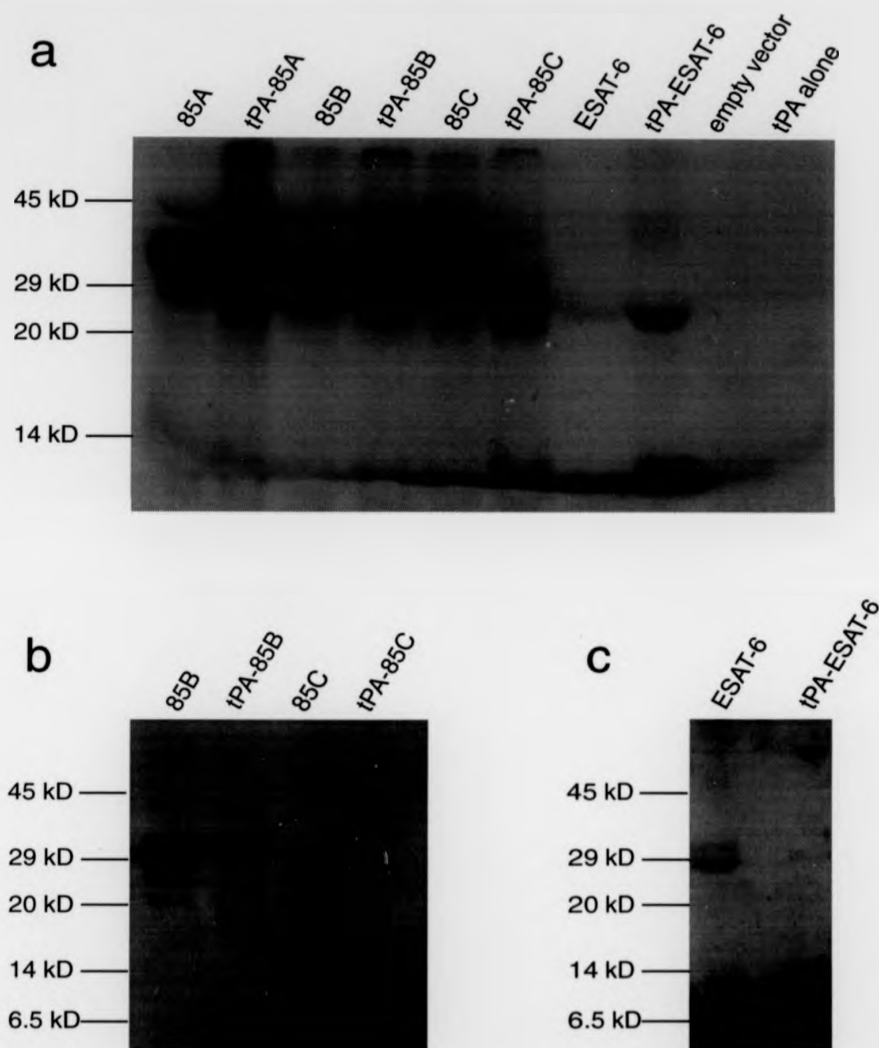


Figure 5.9 *In vitro* coupled T7 transcription/translation of *Ag85* and *esat-6* genes in the absence of microsomes

Transcription/translation was performed using the pWR510 constructs (genes under control of the T7 promoter) in a single step microfuge tube reaction. This resulted in ³⁵S methionine-labelled protein products. These products were then resolved using SDS-PAGE and the signal recorded using autoradiography. **a)** The figure shows products from all the pWR510 constructs. **b)** A detail of a second experiment showing degradation of 85C (third lane from the left). **c)** A detail taken from a third experiment showing the size difference between ESAT-6 and tPA-ESAT-6.

labelled protein products for the whole set of pWR510 constructs. Signals of the correct molecular weight were seen in all lanes except that containing the tPA-85C reaction. Here, there was a slight increase in signal at the gel front, as compared with the two negative controls (empty vector or pWR510-tPA alone). This suggested the possibility of protein degradation. However, the background was relatively high, making overall interpretation difficult. In a previous experiment using the same reaction conditions (which included 85B and 85C constructs only), no signal from the tPA-85C reaction was seen (Figure 5.9b). In this case, the 85C only lane showed a signal of the correct molecular weight, plus a stronger signal which had run with the dye front. Again, this result supported the possibility of the 85C protein being unstable and prone to degradation in this system.

As would be predicted, the presence or absence of tPA resulted in an appropriate difference in molecular weight. This was seen for 85A (Figure 5.9a), 85B in two separate experiments (Figure 5.9a and 5.9b) and ESAT-6 in one of two experiments (Figure 5.9c). In the other ESAT-6 experiment, no apparent difference was seen in the reaction products (Figure 5.9a) due to over-running of the gel.

5.2.2.2 *Transcription/translation in the presence of microsomes*

In order to assess the influence of the signal sequence, canine pancreatic microsomes were included in further reactions. Ultracentrifugation of the reaction products permitted comparison of membrane-associated protein (the pellet) and non-membrane associated protein (the supernatant) (see Section 2.17.2). In addition, further comparisons were made between reaction products with and without microsomes. The results of these experiments are shown in Figures 5.10a (85A), 5.10b (85B and tPA alone) and 5.11a (ESAT-6 and tPA alone).

The figures demonstrate two key features associated with the tPA constructs. These are described below.

1) tPA leads to the association of protein with microsomal membrane

For the membrane-associated fraction (the pellet), the intensity of the band was greater for those constructs which encoded the tPA signal sequence as compared to the same gene product without tPA (see lanes 3 and 6 for Figures 5.10a, 5.10b and 5.11a). This is particularly clear

a

85A constructs						
tPA	-	-	-	+	+	+
microsomes	-	+	+	-	+	+
fraction	na	s/n	pellet	na	s/n	pellet

**b**

	85B constructs						tPA alone construct		
tPA	-	-	-	+	+	+	+	+	+
microsomes	-	+	+	-	+	+	-	+	+
fraction	na	s/n	pellet	na	s/n	pellet	na	s/n	pellet

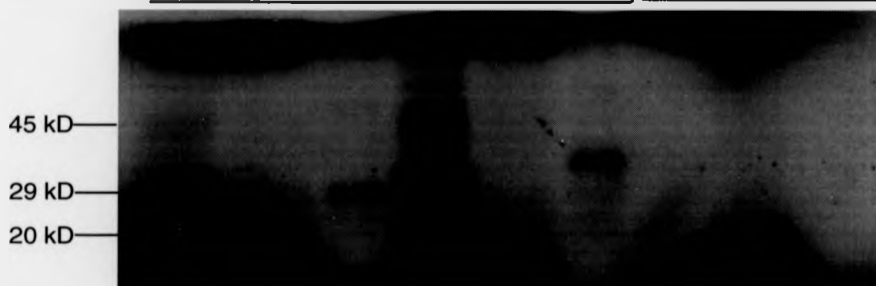
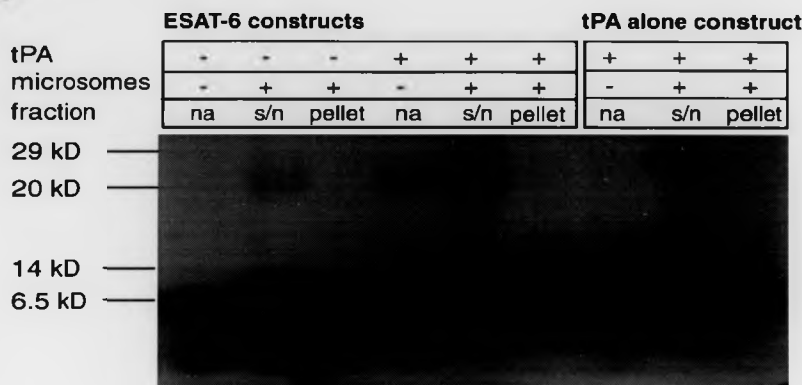


Figure 5.10 *In vitro* coupled T7 transcription/translation of *85a* and *85b* genes with the addition of microsomes - the effect of the tPA signal sequence

Reactions were performed with (+) and without (-) canine pancreatic microsomes. Those reactions in which microsomes were included were subject to ultracentrifugation in order to fractionate the protein products into membrane-associated (pellet) and non-membrane-associated (s/n). **a)** The products from pWR510-85A and -tPA-85A constructs. **b)** The products from pWR510-85B, -tPA-85B and -tPA constructs alone. tPA present= (+) and tPA absent= (-). s/n=supernatant and na=not applicable.

a



b

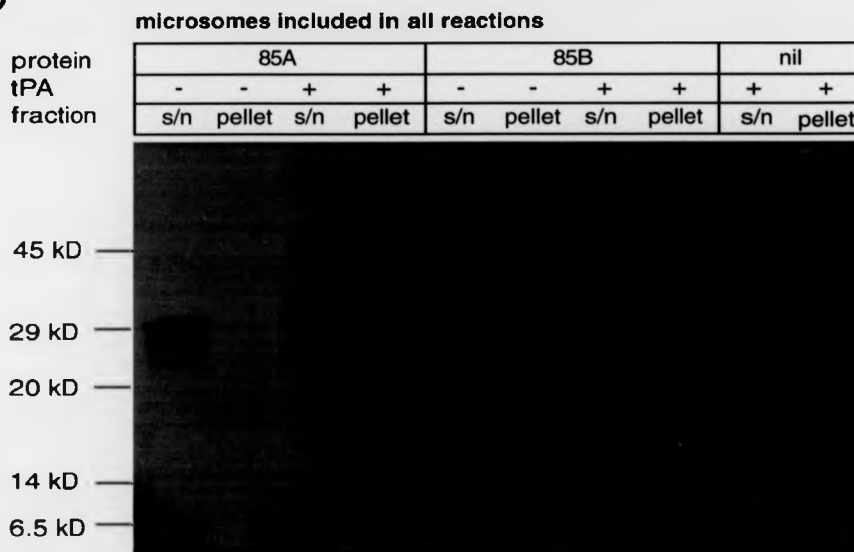


Figure 5.11 *In vitro* coupled T7 transcription/translation of *esat-6* and *Ag85* genes with the addition of microsomes

a) Reactions were performed using pWR510-ESAT-6 constructs both with (+) and without tPA (-). The pWR510-tPA construct was used as a negative control. Canine pancreatic microsomes were added in half of the reactions and the products of these reactions were fractionated into membrane-associated (pellet) and non-membrane-associated (s/n).

b) Products from reactions using the 85A, 85B and tPA constructs were run on a larger gel format in order to improve size resolution. Microsomes were included in all of these reactions. s/n=supernatant and na=not applicable.

in the autoradiograph in Figure 5.11b, where the products of 85A and 85B from the microsome experiment were run in the same gel.

In addition, the relative proportion of product found in the pellet versus the supernatant varied according to the presence of the tPA signal sequence. There was a general trend in favour of a stronger signal occurring with the supernatant fraction for non-tPA constructs and a stronger signal occurring with the membrane fraction for tPA constructs. This provided evidence for membrane-binding of tPA constructs to the microsomes. Good examples of this are shown in Figure 5.11b in: (i) 85A (no tPA) where there is no signal with the membrane fraction (lanes 1 and 2) and (ii) 85B (with tPA) where there is no signal with the supernatant fraction (lanes 7 and 8). This comparison could not be made for *esat-6*, owing to the high background in the low molecular weight region of supernatant fraction. In contrast to the predicted pattern, the 85B (no tPA) reaction showed a definite signal with the membrane fraction. However, there was no increase in molecular weight unlike the relevant tPA counterpart (see below).

2) Evidence in favour of tPA-associated post-translational modification

In the case of the tPA constructs, but not those lacking the signal sequence, there was an increase in molecular weight for membrane-associated proteins (see lane 8, Figure 5.11a) and lanes 4 and 8, 5.11b), as compared with either the supernatant fraction or the products from the reactions not including microsomes. This provides evidence in support of post-translational modification. In addition, two bands were noted for 85A (with tPA) in the supernatant fraction (lane 3, Figure 5.11b). The lower molecular weight band corresponded to the predicted product size (similar to 85A (no tPA) and was smaller in size than the glycosylated 85A (tPA)). The higher molecular weight band migrated a shorter distance than the tPA membrane-associated product (lanes 3 versus 4). Possible reasons for this are discussed below (Section 5.3.1.3).

5.3 DISCUSSION

Following the failure to observe protein expression from the first rVV construct (pSC11-derived rVV-ESAT-6, chapter 3), three strategies to enhance expression were pursued: the addition of alternative genes (Ag85 genes), the use of the T7 promoter and the addition of a signal sequence. The following section discusses the achievements, failures and validity of these strategies.

5.3.1 Ag85 protein expression

In contrast to the pSC11-derived rVV-ESAT-6 immunoblots shown in chapter 3, 85A and 85B rVV-encoded proteins were readily identified. Immunoblotting demonstrated expression using two different monoclonal antibodies (TD-17 and TD-24), a rabbit polyclonal antibody (K92) and serum samples from mice vaccinated with Ag85 DNA constructs. Furthermore, integrity of the coding region was supported by *in vitro* coupled T7 transcription/translation for all of the genes, except for the 85C construct with tPA.

5.3.1.1 Monoclonal immunoblotting of 85A and 85B

As would be expected, the banding pattern was similar for all blots, irrespective of the antibody probe used. However, different antibodies produced marked differences in signal intensity, a feature most pronounced when comparing the mAb, TD-17, with the polyclonal antibody, K92 (Figure 5.3).

In the early part of the study, only mAbs TD-17 and TD-24 were available. According to a previous study, native 85A, 85B and 85C were each recognised by these monoclonal antibodies with a similar degree of antibody reactivity (Drowart et al. 1992). However, in this study, TD-24 was unable to identify rVV-encoded 85B (data not shown) and TD-17 could only recognise protein from the rVV-85B constructs following prolonged autoradiographic exposure (Figure 5.2b). Three possible explanations were considered: (i) only small amounts of recombinant 85B were made; (ii) the mAbs recognised native 85B more readily than the recombinant counterpart (perhaps due to a structural difference or post-translational modification); and (iii) despite the previous report, the mAbs recognise 85B less clearly than 85A. Figure 5.2a(ii) shows a Western blot probed with mAb TD-24, which included an MTB CF control lane. This mAb was able to identify two bands in the MTB CF lane, a more intense 30 kDa band (consistent with native 85B) and a less intense 32 kDa band (consistent with 85A). This finding is consistent with the fact that 85B is known to predominate in MTB CF (Drowart et al. 1992) and suggests that mAb TD-17 can recognise 85B, at least in its native secreted form. An argument against the first explanation (less recombinant 85B as compared with 85A), comes from the dot blot experiments. These showed no difference in levels of expression for either of the proteins with and without tPA (Figure 5.1 and two repeat dot blots, data not shown). Thus, it was proposed that the pattern of mAb recognition of 85A and 85B may have been due to conformational or

glycosylation differences. This could also explain the disparity between dot blots (undenatured protein) and Western blots (protein subjected to SDS-PAGE).

5.3.1.2 *Recombinant 85C expressed in mammalian cells - problems of glycosylation*

Given the difficulty with mAb recognition of 85B, it was proposed that glycosylation might account for the failure to identify rVV-derived 85C. In support of this hypothesis, the existing mAbs were generated using the native mycobacterial Ag85 proteins. Whilst evidence exists for glycosylation of other secreted mycobacterial proteins such as the 19 kDa and 45 kDa MTB proteins (Garbe et al. 1993; Dobos et al. 1995), there has been no data to suggest glycosylation of native Ag85 proteins (Dr C. Abou-Zeid, personal communication). If these Ag85 proteins were to be expressed in a eukaryotic system, they could be subject to post-translational modification. From sequence data, potential glycosylation sites for N-linked glycans include -Asn-Xaa-Ser/Thr- (where Xaa is any residue) and for O-linked glycans, Pro/Ser/Thr-rich regions (Carlsson, 1993). In the case of Ag85 complex proteins, the number of theoretical sites is high for 85C (seven N-linked sites), low for 85A (one N-linked site) and somewhere between for 85B (four N-linked sites). Thus, eukaryotic expression, using systems such as transfected cells, DNA vaccination or rVV, could give rise to glycosylated products that may not exist when derived from mycobacteria. This would be particularly true for those proteins which include a eukaryotic signal sequence. This would facilitate translocation into the endoplasmic reticulum and progression through the Golgi system.

The problem of glycosylated protein recognition was noted using recombinant Ag85 expressed in transfected eukaryotic cells (Dr M. Liu, Third International Conference on the Pathogenesis of Mycobacterial Infections, Stockholm, June 1996). Probing for recombinant Ag85 proteins using the available mAbs produced a strong signal for 85A, a weaker one for 85B and an absent one for 85C. However, recombinant 85C was subsequently identified using serum from 85C DNA vaccinated mice or polyclonal antibody serum. Moreover, a high degree of glycosylation was noted with this particular protein, as demonstrated by graded deglycosylation. In this study, a similar pattern of mAb reactivity was noted for the three rVV-generated Ag85 proteins, a strong signal for 85A, a weak one for 85B and an absent signal for 85C. Therefore, sera from Ag85 DNA vaccinated mice were obtained in order to probe rVV-Ag85 Western blots. Both 85A and 85B were recognised with a

banding pattern not dissimilar to that obtained with the mAb TD-17. Unlike Ag85 from transfected cells, 85C was not identified (Figures 5.4 and 5.5). At a later date, the same proteins were probed with the rabbit polyclonal antibody, K92. This demonstrated recombinant products from both 85A and 85B constructs, but again no signal from 85C was obtained (Figure 5.3a).

5.3.1.3 *Evidence for glycosylation of rVV-Ag85 - immunoblotting and in vitro transcription/translation*

Western blotting of the rVV Ag85 constructs showed multiple bands for both tPA-85A (two bands) and tPA-85B (three bands) (Figure 5.3). The molecular weight for these antibody-reactive targets is greater than would be predicted from their amino acid sequences. This suggests post-translational modification and that each band represents a different glycoform of the same protein.

Evidence in support of glycosylation comes from the constructs incorporating the tPA signal sequence. Firstly, N-glycosidase treatment of rVV-encoded tPA-85B resulted in the replacement of the three higher molecular weight bands with two bands of lower molecular weight. This is consistent with partial deglycosylation (Figure 5.6). Secondly, the addition of microsomes to the *in vitro* transcription/translation expression system resulted in products of larger molecular weight and, in most cases, these were associated with the membrane fraction (Figure 5.10 and 5.11). There was no evidence in favour of glycosylation for the non-tPA constructs.

Using the *in vitro* transcription/translation system with microsomes, an interesting finding was noted with the tPA-85A construct. Two strong, discrete signals were noted in the supernatant fraction. The low molecular weight band was consistent with unmodified protein. The second band was of a higher molecular weight than the membrane-associated fraction from the same reaction (Figure 5.11b). One could explain this finding in terms of the degree of glycosylation, with a more fully glycosylated product being 'secreted' back into the supernatant. However, further work would be required to confirm this explanation.

5.3.1.4 *Protein expression using the T7 as compared to the p7.5 promoter*

rVVpT7 constructs included the *85b* and *85c* genes both with and without tPA. Similarly to the rVVp7.5 constructs, only 85B was identified and expression was greater for tPA-85B

than for 85B alone. In addition, the same banding pattern was noted for these constructs. Surprisingly, levels of protein expression were less than for the early/late promoter (Figure 5.7). This is in stark contrast to the reported literature which states that a 10-50 fold increase in protein expression can be obtained using the T7 promoter. Indeed, in one case, the foreign gene product accounted for 10% of the total cell protein (Elroy-Stein et al. 1989). Possible explanations for the failure to elicit high protein levels using the T7 promoter include: (i) inaccurate quantification of viral titres, thus limiting the proportion of cells which are dually infected (a pre-requisite for recombinant protein expression); (ii) a problem with the rVV encoding T7 polymerase (vTF7-3); and (iii) a problem with the rVV encoding the foreign genes under the T7 promoter. In order to reduce the possibility of inaccurate viral titres, a fresh stock of vTF7-3 was made and the pfu/ml re-assessed. In addition, the rVVpT7-Ag85 constructs were also re-titred. The second stock of vTF7-3 was taken from the laboratory of Dr U Gompels, LSHTM, and was known to work in conjunction with plasmids containing human herpes virus-6 genes (Dr R Anderson, personal communication). Despite these measures, no difference in expression was noted. The third potential problem could be due to the transfer plasmid, pWR510. In theory, this plasmid contains the necessary elements for high level protein expression: the T7 promoter, the encephalomyocarditis untranslated 5' leader (an internal ribosomal entry site permitting cap-independent translation) and the T7 transcription terminator. However, no quantitative data has been published concerning pWR510-derived rVV, as compared with other rVV using vaccinia promoters. In addition, a mutation within one of the elements responsible for high level expression might have occurred.

It was not relevant to pursue the problems encountered with this system. A key reason for utilising the T7 promoter was to identify recombinant protein which was undetectable using the p7.5 promoter. However, 85A and 85B proteins were readily identified using the vaccinia promoter and 85C was not detected using either of the promoters.

5.3.2 ESAT-6 protein expression

5.3.2.1 *tPA and T7 strategies*

In the second generation of rVV, an ESAT-6 construct was made to include the tPA signal sequence. It was hoped that addition of this region would stabilise the protein, allow translocation into a membrane compartment and permit a readout using the available mAb. Despite these measures, no signal was identified. In addition, the rVVpT7-ESAT-6

constructs were non-functional due to a cloning error (see Section 4.3.2.2), preventing assessment of expression using the vaccinia/T7 hybrid system. This latter problem could have been addressed, if the frameshift error found in the initial PCR-generated pWR510-ESAT-6 and -tPA-ESAT-6 had been corrected by subcloning the gene from p1108-tPA-ESAT-6. However, construction of further rVV-ESAT-6 did not seem appropriate, as the T7 system had failed to produce a high level of expression for the 85B constructs.

5.3.2.2 *In vitro* transcription/translation - evidence for glycosylation of tPA-ESAT-6

Strong signals of the correct molecular weight were seen with the sub-cloned pWR510-ESAT-6 constructs (see Table 4.1, plasmids P23 and P22) demonstrating integrity of the open reading frame (Figure 5.9a and 5.9c). Truncated products were noted using the initial PCR constructs, pWR510-ESAT~~del~~-6 (P5) and pWR510-tPA-ESAT~~del~~-6 (P11), consistent with premature stop codons introduced by a frame shift (data not shown). Following the addition of microsomes, there was strong evidence for post-translational modification of ESAT-6 (Figure 5.11a). In lane 6, the membrane-associated tPA-ESAT-6 fraction, two bands are present, a faint band of lower molecular weight comparable with the 'no microsomes' tPA-ESAT-6 reaction and a stronger band of higher molecular weight. Unfortunately, the background signal obliterated the signal from the supernatant fraction. However, one might predict that the band would correspond to the 'no microsome' lane.

There are two potential N-glycosylation sites on ESAT-6. Studies of native ESAT-6, using a glycan-protein double binding system, showed no evidence of glycosylation (Sorensen et al. 1995). It is tempting to speculate that failure to identify rVV-encoded tPA-ESAT-6 might have been due to the presence of a glycan interfering with antibody binding. However, it would also be necessary to suggest that the non-tPA ESAT-6 was either rapidly degraded or also glycosylated (not suggested from the *in vitro* transcription/translation data). This hypothesis could be verified, either by deglycosylation of the rVV-tPA-ESAT-6 cell lysate, as described in Section 2.16.3.8 (plus additional enzymes if required), or using an alternative antibody probe which was not available at the time this work was carried out.

5.3.2.3 *Capture ELISA for ESAT-6*

Given the potential value of ESAT-6 as a key candidate antigen for the development of a sub-unit vaccine, Prof M. Harboe and colleagues, in Oslo, developed a quantitative assay

for identification of recombinant ESAT-6 (see Section 5.2.1.7) (Harboe et al. 1997). In addition to the mAb HYB 76-8 (first layer), this assay made use of a polyclonal anti-peptide antibody (third layer). This latter reagent was identified by predicting candidate B cell epitopes from an ESAT-6 hydrophilicity plot. Three candidate peptides were selected from outward facing, hydrophilic regions of the molecule which would be expected to contain surface-exposed areas of the polypeptide chain with likely B cell epitopes. These were synthesized, linked to keyhole limpet haemocyanin and used repeatedly to immunise rabbits. The immune sera were then tested for reactivity against the immunising peptide and ESAT-6. A 23mer peptide, p40-62, was particularly effective and this was used in the final assay. The capture ELISA was then able to quantitate both native and recombinant ESAT-6 from different sources, including rVV-ESAT-6 cell lysates obtained from this study. A very high level of ESAT-6 was identified from rVV-tPA-ESAT-6. A much more modest but definite signal was identified from rVV-ESAT-6 without the signal sequence (Figure 5.8) (Harboe et al. 1997). This result is somewhat surprising, as the initial capture antibody, HYB 76-8, was the same mAb used in the immunoblotting experiments performed in this study, where no signal was identified. This result could be explained in two ways: (i) the capture ELISA is more sensitive than the immunoblotting; or (ii) the binding of HYB 76-8 to rVV-generated ESAT-6 may behave differently in the ELISA, as compared with the immunoblot assay. In the former, the mAb is bound to plastic and the antigen is in liquid phase, in the latter, the converse is true, with the denatured antigen bound to nitrocellulose membrane and the mAb in solution. The first explanation is unlikely as the chemiluminescent protocol used for immunoblotting permitted detection of ESAT-6 from 100-fold diluted MTB CF (estimated sensitivity of ≤ 1.6 ng, see Figure 3.9), as compared with undiluted total protein rVV cell lysates. In the ELISA, the concentration of ESAT-6 in the MTB culture filtrate was only ~15-fold greater than the rVV-tPA-ESAT-6 cell lysate (see Figure 5.8).

5.4 CONCLUSION

Following the failure to identify ESAT-6 from the first rVV, a second generation of rVV were constructed. Two of the three strategies to obtain rVV expression of MTB secreted proteins were successful. The introduction of the *Ag85* genes under the control of the vaccinia early/late p7.5 promoter resulted in readily identifiable expression of both 85A and 85B. Construction of a signal sequence fusion further facilitated expression of both of these proteins. Whilst immunoblotting failed to identify rVV expression of ESAT-6, a capture ELISA showed clear expression of this protein with a particularly strong signal obtained

from the tPA fusion. The addition of this tPA signal sequence led to microsomal membrane association and post-translational glycosylation for 85A, 85B and ESAT-6, as evidenced by *in vitro* coupled T7 transcription/translation. Further evidence was provided by successful N-deglycosylation of three 85B glycoforms seen in rVV-tPA-85B. The third strategy employing the T7 promoter was unsuccessful. The results from the ESAT-6 capture ELISA suggested that failure to detect this recombinant protein by immunoblotting may have been due to denaturation of a conformational epitope recognised by the mAb rather than the lower sensitivity of Western blotting.

6 Functional application of rVV

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6.1 INTRODUCTION

The purpose of rVV-Ag85 and -ESAT-6 construction was to assess both human CD8⁺ CTL function and vaccine efficacy in murine protection models. This chapter describes the preliminary data towards achieving these goals. The first part of the chapter covers human CTL. These assays were performed as part of this study using live BCG as a means to restimulate CTL. In addition, parallel CTL assays were performed by others in Prof A. Hill's laboratory, Institute of Molecular Medicine, Oxford using a different system (peptide restimulation of CTL).

Murine immunological studies were performed in Dr K. Huygen's laboratory, Pasteur Institute, Brussels (using the rVV Ag85 constructs) and in Dr P. Andersen's laboratory, Statens Seruminstitut, Copenhagen (using the rVV ESAT-6 constructs). In addition to the CTL experiments performed in this study (Sections 6.2.1 and 6.2.2), this chapter discusses data from the three collaborating laboratories (Section 6.2.3 and 6.2.4) in order to show the functional application of the constructs.

6.1.1 Human CTL work

An increasing body of evidence has favoured a role for MHC class I-restricted T cells in protective immunity (Section 1.3.2). No mycobacterial antigens are known to be recognised by this population of cells, although there is indirect evidence in favour of Ag85 recognition (Rees et al. 1988; Manca et al. 1991). This laboratory had previously developed a system for stimulating human CD8⁺ CTL from TB patients and BCG vaccinees, using live BCG (Turner and Dockrell, 1996). As ESAT-6 is absent from BCG (Harboe et al. 1996) and Ag85A is the most abundant of the three Ag85 proteins in BCG CF (Drowart et al. 1992), the rVV-Ag85A constructs were selected as the initial recombinants for infection of the CTL target cells. The levels of protein expression were higher for rVV-tPA-Ag85A, as compared with the same construct lacking the signal sequence. However, from a theoretical perspective, the recombinant protein lacking the signal sequence would be more prone to cytoplasmic degradation, resulting in more efficient MHC class I processing and presentation to CD8⁺ CTL. Therefore, it was thought appropriate to assess both rVV-Ag85A constructs, with and without the tPA leader sequence.

In this study, live BCG was used to restimulate CTL derived from BCG vaccinated healthy donors and rVV-Ag85A constructs were used to infect autologous cultured macrophage

target cells in a standard 6 hour ^{51}Cr release CTL assay. In preparation for assessing ESAT-6 specific CTL, several strategies were pursued to restimulate this sub-population. These included culture of *M. marinum* (MM) and verification of ESAT-6 expression and coating 1 μm latex beads with soluble antigen containing ESAT-6 (ST-CF from the Statens Seruminstitut, Copenhagen). It was intended that re-stimulating CTL with either live MM or antigen-coated beads, would lead to expansion of a putative ESAT-6 CTL population. Previous data demonstrated that ESAT-6 was only expressed by a few mycobacteria including the highly pathogenic *M. tuberculosis* complex species and the less pathogenic MM (Sorensen et al. 1995). This suggested the possibility of using MM, a Category II organism requiring less stringent safety precautions during the seven day T cell restimulation. Evidence existed for increased class I processing of both MTB and BCG antigens when macrophages were infected with live rather than dead organisms (Mazzaccaro et al. 1996; Turner and Dockrell, 1996). It seemed reasonable to assume that MM would behave in a similar manner, particularly as this organism behaves in a similar way to MTB with respect to its cellular trafficking - avoidance of the host cell endocytic pathway and prevention of phagosomal acidification (Barker et al. 1997). An alternative strategy was to use antigen-coated beads (discussed in Section 1.3.2.7). Class I processing of antigen coated to latex beads had been demonstrated for ovalbumin (Kovacsovics Bankowski et al. 1993; Harding and Song, 1994). Therefore, CF samples containing ESAT-6 were covalently linked to latex beads for restimulation of putative ESAT-6 specific CTL.

In Oxford, collaborators carried out peptide restimulation of putative CTL derived from TB patients and healthy controls. They used a panel of ESAT-6 and Ag85 peptides congruent with HLA class I allele-specific motifs. rVV-ESAT-6 made in this study was used to demonstrate that peptide-stimulated CD8⁺ T cell clones recognised target cells which endogenously processed whole recombinant antigen as well as peptide-pulsed target cells.

6.1.2 Mouse immunology - background

Collaborators in Brussels and Copenhagen each made use of established murine models of mycobacterial infection. In Brussels, a BCG infection model had been established to assess the cytokine response from immune spleen cells to recall antigens such as BCG, PPD and Ag85A (Huygen et al. 1992). Previously, in the same laboratory, Ag85 DNA vaccinated mice had been assessed for humoral and T cell responses following immunisation. In addition, protection was measured using an intravenous BCG challenge of 2×10^6 CFU,

given 3-10 weeks after the last vaccination. Protective efficacy was measured according to the reduction in CFU counts of spleen and lung homogenates, taken at several time points post-challenge (Huygen et al. 1996). In Copenhagen, a similar protection model was used. The main differences between the two models were that the Copenhagen group: (i) challenged with a lower dose of mycobacteria (5×10^4 CFU); (ii) challenged with virulent MTB (H37Rv) in some cases; and (iii) gave immunised mice a prolonged rest (at least 12 weeks) prior to challenge (Andersen, 1994). This latter issue of resting the immunised mice permitted a clearer distinction between protection afforded at the time of (or shortly after) the primary immune response, as compared with longer-lived protection mediated by immunological memory. Neither group has used aerosolised challenge with live mycobacteria. Both the Pasteur Institute, Brussels and the Statens Seruminstitut were provided with cushion-purified rVV for the mouse protection studies.

6.2 RESULTS

Sections 6.2.1 and 6.2.2 include human CTL work performed in this study. Sections 6.2.3 and 6.2.4 represent work performed by collaborators who have kindly given permission to present the human peptide-stimulated CTL data and murine immunology. Although these latter data were not generated in this study, their inclusion shows the functional application of rVV and permits further discussion.

6.2.1 Human CTL - a whole antigen approach

6.2.1.1 *Is rVV-encoded Ag85A expressed in 7 day cultured primary human macrophages?*

Clear evidence for expression of rVV-Ag85, 85B and ESAT-6 had been demonstrated in infected TK-143 cells (Section 5.4). However, the same expression is not necessarily true for rVV infection of primary human macrophages, the cells used as CTL targets in this laboratory (Turner and Dockrell, 1996). Broder and colleagues had previously demonstrated that not all rVV recombinant proteins were expressed in human seven day cultured macrophages (1994). Recombinant protein was expressed only when the foreign gene was under the control of an early but not a late promoter. Importantly, with respect to the rVVpT7 constructs developed in this study, the researchers noted that primary cultured macrophages permitted high level expression using the vaccinia/T7 hybrid system, in contrast to absent expression with late promoters.

In theory, the early component of the p7.5 promoter should permit rVV expression in infected macrophages. This was confirmed using an immunofluorescence technique, as described in Section 2.16.4. Figure 6.1 shows immunofluorescence of rVV-infected primary human macrophages. rVV-tPA-Ag85A expression was compared with rVV-tPA alone. The propidium iodide stain shows the macrophage nuclei (blue fluorescence) indicating all the macrophages present in the field. The green signal represents binding of FITC-coupled secondary antibody to the anti-Ag85 mAb, TD-17, indicating expression of Ag85A. No fluorescence was seen in the negative control (rVV-tPA infected macrophages). A clear signal was present in the rVV-tPA-Ag85 infected macrophages. The fluorescence was suggestive of a cytoplasmic distribution, with a few additional punctate areas of increased signal, perhaps representing accumulation of Ag85A following translocation into a vacuolar compartment. Approximately 25% of the macrophages showed immunofluorescence using a moi of 25:1 (not shown in figure). No difference in signal was noted using rVV-Ag85A without the tPA signal sequence (data not shown).

6.2.1.2 *Macrophage infection with two different batches of BCG*

Our laboratory had previously noted a batch to batch variation between different cultures of BCG in their ability to activate CD8⁺ T cells. These cultures had been derived from the same source (freeze-dried BCG Glaxo, Evans Medical) and cultured under the same conditions (grown in Middlebrook 7H9/OADC and harvested during log phase growth). For example, others in our laboratory had previously grown up and assessed two batches of BCG: JT96023 (6.1×10^7 CFU/ml) and JT96010 (7.7×10^8 CFU/ml). In this study they are designated as batch-1 and batch-2 respectively. Under similar experimental conditions, each batch was used to stimulate PBMC from BCG vaccinees. Activated CD8⁺ T cells (CD25⁺) and CTL readouts gave a different pattern of results for each batch, as measured by flow cytometry and the ⁵¹Cr release assay; batch-1 required approximately a log₁₀ lower moi to achieve a similar CTL assay result (J. Turner, personal communication). As it was possible that the differences observed reflected batch variation in bacterial viability, this issue was assessed as described below.

Mycobacterial CFU counts were repeated using randomly selected frozen aliquots of BCG (Section 2.18.2.1). For batch-2, an average of two experiments showed just over half a log decrease in CFU as compared with the initial count following culture (7.7×10^8 CFU/ml in May 1996 fell to 1.6×10^8 CFU/ml in November 1996). This was consistent with a similar

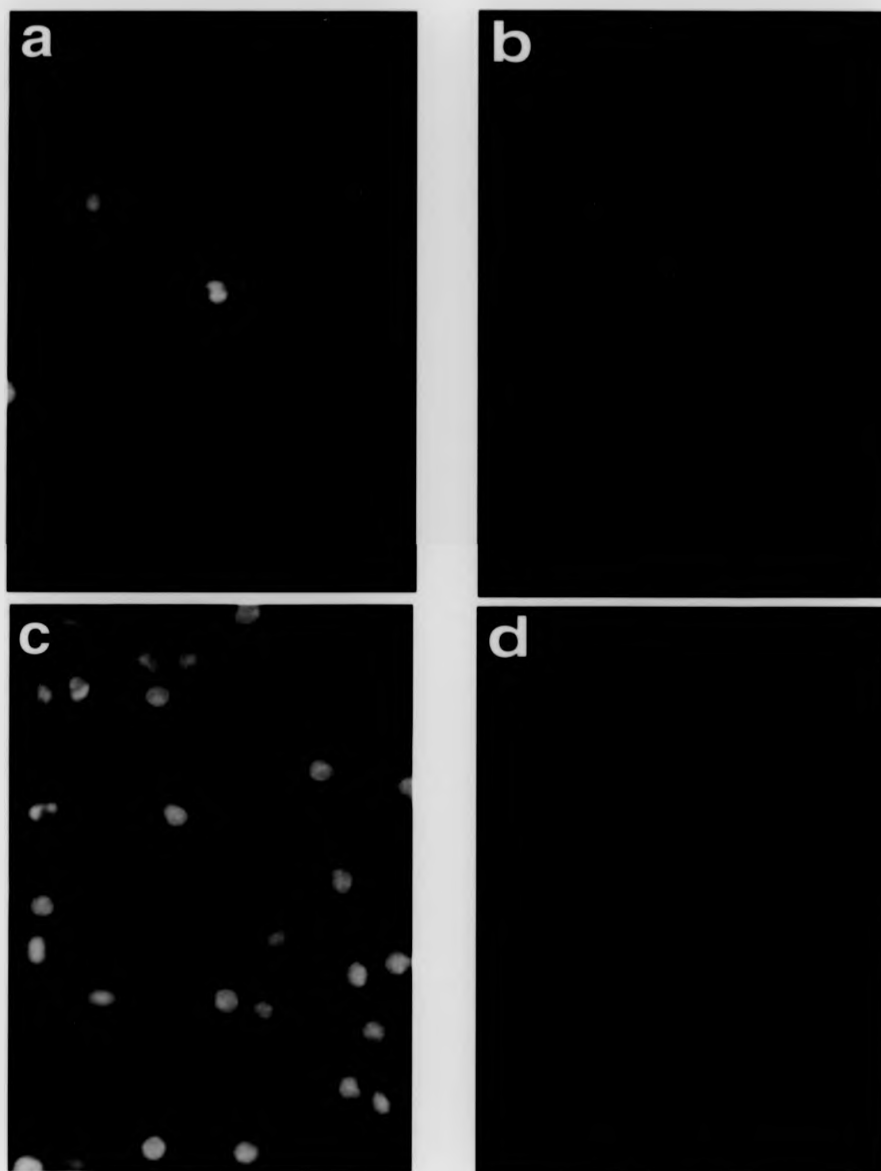


Figure 6.1 **Immunofluorescence of rVV-infected macrophages**

Top images: rVV-tPA-Ag85A infection. Bottom images: rVV-tPA infection
a) & c) show staining of macrophage nuclei with DAPI.

b) & d) show immunofluorescence of rVV-infected macrophages using the
Ag85-specific mAb, TD-17 and a FITC-conjugated secondary antibody.

fall in viability over time previously noted for batch-1 BCG (Dr J. Turner, personal communication).

Both batches of BCG were used in an overnight infection of seven day primary human macrophage cultures, as described in Section 2.18.2.2. The tissue culture eight chamber slide system permitted infection of the same macrophage monolayer using the two batches of BCG, each at four different moi's (25:1, 5:1, 1:1 and 0.2:1). Following methanol fixation, the upper 'chamber' section was removed from the glass slide and the infected macrophage monolayer was stained using the Ziehl-Neelsen (ZN) stain. This permitted uniform staining of the entire monolayer. The two batches of BCG-infected macrophages were assessed using light microscopy. In the case of the 5:1 moi, batch-1 BCG was taken up by virtually all of the macrophages whilst batch-2 BCG was identified in only approximately 50% of macrophages. Figure 6.2 shows both batches of BCG following macrophages infection (moi 5:1) and ZN staining. The morphological appearance and cellular distribution of the two batches of BCG was different. Infection with batch-1 BCG showed fragmented bacilli, with variable uptake of the acid fast stain. Moreover, the bacilli were fairly evenly distributed throughout each cell. In contrast, batch-2 BCG infection showed the bacilli to be more localised to one region within the cell. Furthermore, the evidence for 'cording' (the formation of cord-like structures as a consequence of cord factors (Besra and Chatterjee, 1994) was seen more clearly. In addition, acid fast staining of batch-2 bacilli was both more intense and homogenous with less overall fragmentation.

6.2.1.3 CTL assay - optimisation of BCG moi of macrophages

A CTL assay was employed in order to identify BCG-stimulated human CTL from BCG vaccinees. BCG batch-1 and -2 were utilised in separate CTL experiments. Batch-1 had previously been optimised by Dr J. Turner who had identified the ideal moi required for effector cell restimulation which was either 0.2 or 1 bacillus:macrophage and for target cell infection was 1 bacillus:macrophage. Similar optimisation experiments using batch-1 BCG were repeated in order to demonstrate reproducibility of the assay for use in this study. The ^{51}Cr -release assay results obtained using batch-1 BCG-stimulated CTL, derived from two healthy BCG vaccinees are shown in Figure 6.3. In this experiment, a single moi for effector restimulation and target infection was selected (1:1 for each subject). This was based on previous data (Turner and Dockrell, 1996). Following one week of *in vitro* BCG-stimulation, the effector cells were added at three effector:target ratios: 25:1, 5:1

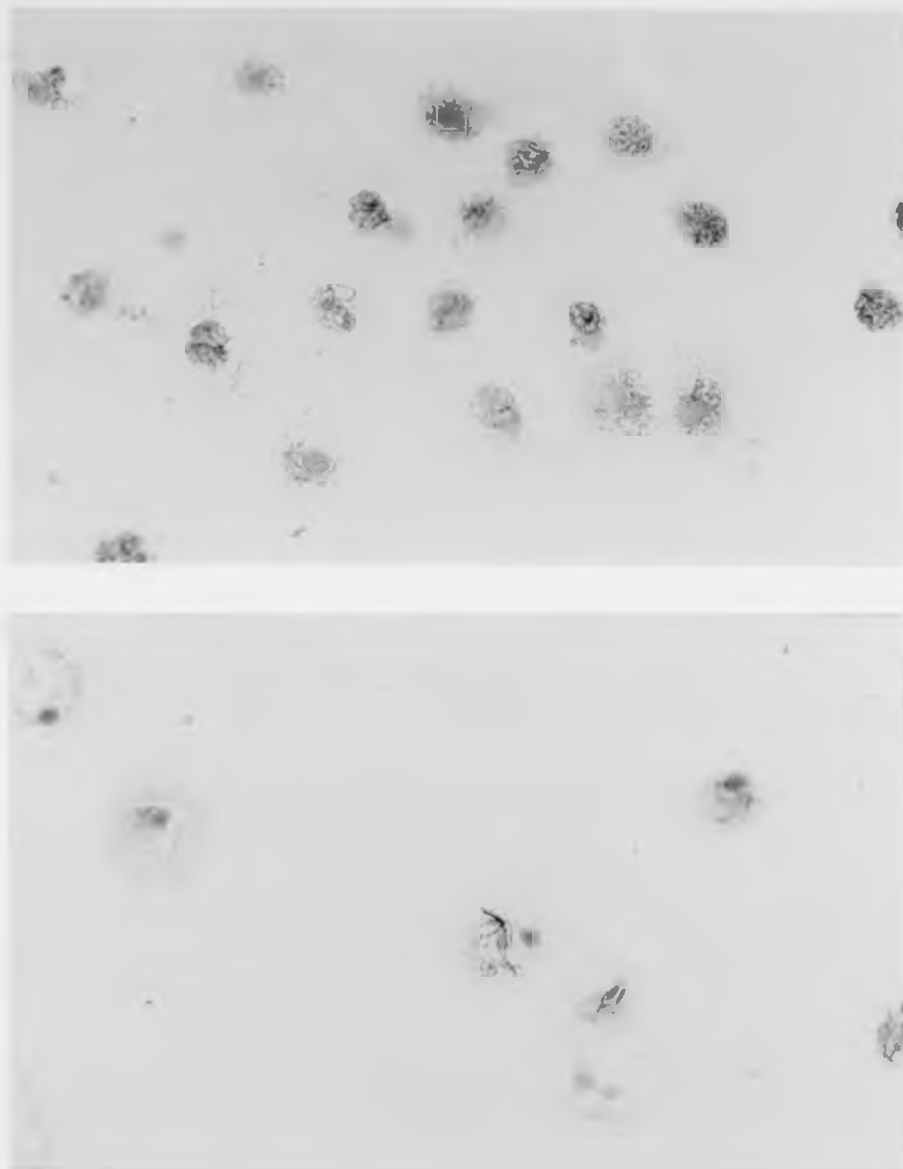


Figure 6.2 Ziehl-Neelsen staining of BCG-infected macrophages using two different batches of BCG

Two batches of BCG were used to infect macrophages at four moi's (25:1, 5:1, 1:1 and 0.2:1). Following overnight incubation with BCG, the macrophages were fixed and stained using the Ziehl-Neelsen technique. The figure shows photographs of macrophages infected with one of the BCG batches (batch-1=top photograph, batch-2=bottom photograph), using an moi of 5:1 in each case. Photographs were taken under the x40 objective.

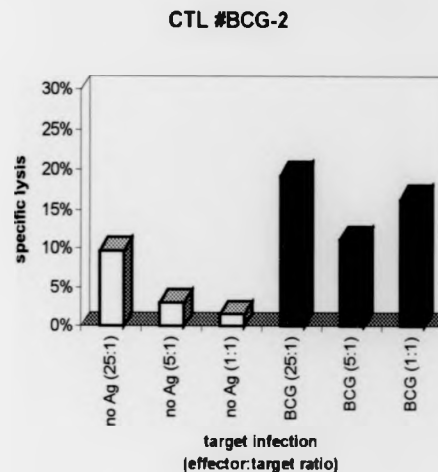
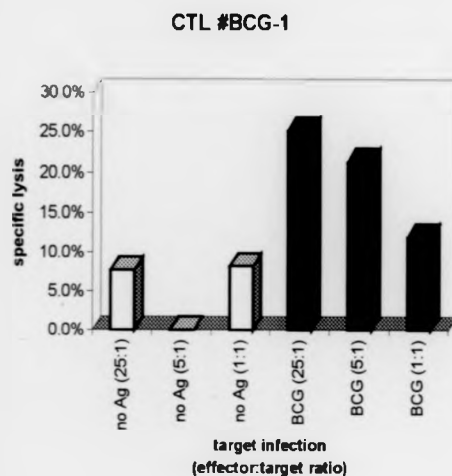


Figure 6.3 ^{51}Cr release CTL assays using BCG-stimulated PBMC as effector cells and BCG-infected macrophages as target cells
 CTL assays were performed using BCG-infected PBMC derived from two healthy, BCG vaccinated, PPD skin-test positive donors. Effector cells were generated, using 7 days of *in vitro* restimulation of CTL with live BCG (batch-1) at a moi of 1:1. Autologous macrophage targets were infected with BCG (moi of 1:1) and a standard 6 hour ^{51}Cr release assay was performed. Three effector target ratios were assessed (25:1, 5:1 and 1:1) for 'no Ag' targets and 'BCG-infected' targets.

and 1:1 (see Section 2.18.1 for methodology). Specific lysis varied from 12-26% for all target cells pulsed with BCG and was less than 10% for all the 'no Ag' target cells. An effector:target ratio dose effect was observed for BCG-pulsed target cells for the assay #1. This was less apparent for assay #2. The converse was true for the 'no Ag' control target cells where an effector:target ratio dose effect was observed in #2. This latter dose effect could be attributed to non-specific killing from lymphocyte-activated killer (LAK) cells. The supply of batch-1 BCG was limited, and therefore further optimisation experiments using batch-2 BCG were performed. The data generated produced results which conflicted with the optimum moi's of batch-1 BCG. These data suggested that using batch-2 BCG either a target cell moi of 5:1 or 25:1 was required (see below). Whilst the higher moi produced marked specific lysis on occasions, this was not reliable and in some cases, a moi of 25:1 resulted in significant macrophage death (data not shown). Thus, subsequent experiments using batch-2 BCG, moi's of 1:1 for effector stimulation and 5:1 for target cell infection were selected.

6.2.1.4 *rVV-tPA-Ag85A infection of target macrophages*

Sixteen CTL assays were performed, usually in pairs. PBMC were derived from healthy BCG vaccinees who were known to be PPD skin test positive. Constructs expressing Ag85A (with and without the signal sequence) were used to infect target cells at either a single moi (25:1) or a range of moi's (25:1, 5:1 and 1:1). A 'no Ag' negative control and a BCG-infected positive control were included in every case. CTL assays were considered to have yielded significant results only when the negative control was less than 10% specific lysis and the positive control greater than 10% specific lysis. Results failing to meet these criteria were disregarded. Data which generated negative specific lysis results were treated as a zero result (i.e. where the background isotope release from macrophages was greater than the isotope release from macrophages with effectors - see equation in Section 2.18.1). This applied to some of the negative controls and in the data presented, was never greater than -10%. If the criteria was met for the positive and negative controls, then the experimental arms were deemed to show specific lysis if the lysis was above 10%. Of the sixteen CTL assays performed, four assays fulfilled the criteria for positive CTL induction. Figure 6.4 demonstrates two assays comparing rVV-tPA-Ag85A-infected target cells with the rVV negative control. Specific lysis of Ag85A expressing target cells was 22 and 11% respectively, whilst both the rVV negative controls were under 5%. In addition, the assay, CTL #4, showed a moi dose effect for the BCG positive control.

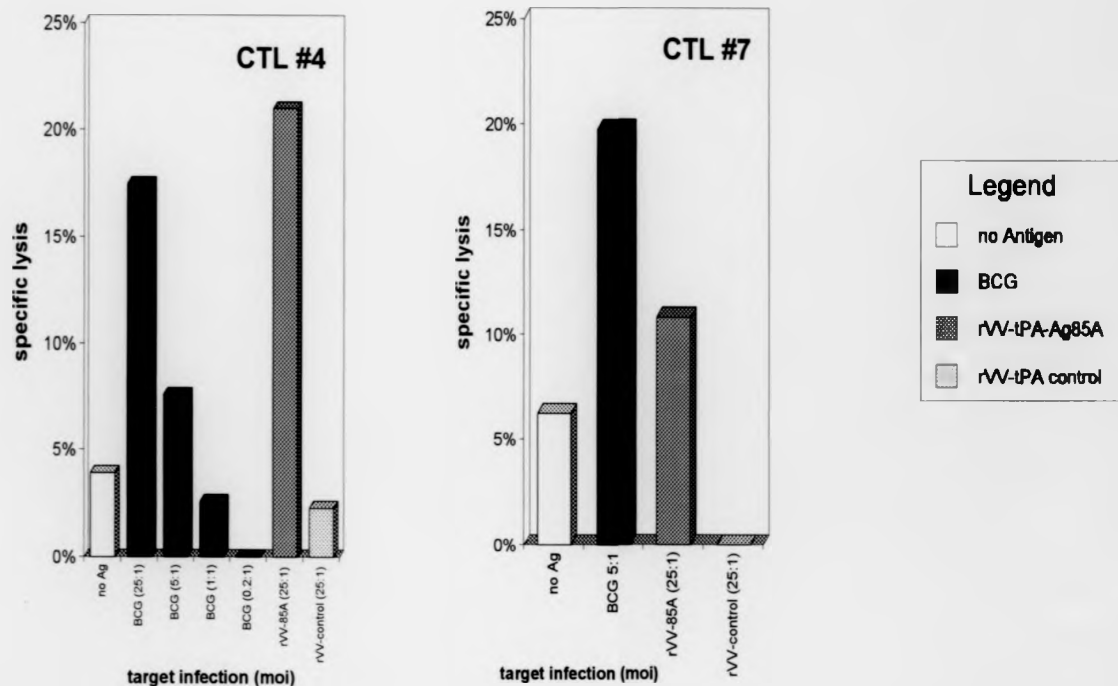


Figure 6.4 CTL assays using rVV-infected macrophage target cells

CTL assays were performed using PBMC from two healthy BCG-vaccinated subjects. BCG was used to restimulate CTL using a moi of 1:1. rVV was used to infect macrophage targets. BCG infected macrophage targets were used as a positive control. Both assays show a comparison between an rVV expressing Ag85A and an rVV control lacking the gene insert (rVV target moi of 25:1). In CTL #4, a series of fivefold BCG target moi's were selected (25:1, 5:1, 1:1, 0.2:1) to identify the best moi for the positive control (BCG-infected targets) and to demonstrate a dose effect.

In order to ascertain the optimum rVV:macrophage moi, a subsequent experiment compared three rVV:macrophage ratios: 25:1, 5:1 and 1:1. This is shown in Figure 6.5 where specific lysis was seen for rVV-tPA-Ag85A moi's of 25:1 and 5:1 but not 1:1. The rVV negative controls were all under 5%. A fourth CTL assay showed a similar trend, but whilst specific lysis for rVV-tPA-Ag85A was 22%, the rVV control was 12% (data not shown).

The remaining CTL assays showed either high background 'specific lysis' (4 subjects) and/or the positive control was below the threshold of 10% (9 subjects) (data not shown). These assays included comparisons between tPA-Ag85A and Ag85A alone. Therefore, no conclusions could be made concerning a possible difference between the constructs.

6.2.2 Alternative strategies for in vitro human CTL restimulation

If a whole antigen approach was going to be employed for restimulation of CD8⁺ CTL, BCG could not be used for ESAT-6 detection, as discussed above. MTB could potentially stimulate ESAT-6 specific CTL, but several practical reasons and health and safety issues prevented these experiments from being carried out in the time available for the study. Therefore, two alternative approaches were developed with a view to restimulating putative low-precursor frequency CTL specific for ESAT-6.

6.2.2.1 *M. marinum* - a Category II organism that expresses ESAT-6

In order to confirm that *M. marinum* expressed ESAT-6, both MTB and *M. marinum* CF were prepared as described in Section 2.3.3.2. A single culture of MTB was prepared by growing the bacteria in Middlebrook 7H9/OADC medium. Several cultures of MM were prepared, which included growth in 7H9/OADC, 7H9/ADC and Sauton's media. Figure 6.6 shows dot and Western blots of different types of CF: (i) MM CF generated in this study (grown in 7H9 medium plus either OADC or ADC supplement or Sauton's medium); (ii) MTB CF from Copenhagen (grown in Sauton's medium); (iii) BCG CF from Brussels (two batches grown in Sauton's medium). The immunoblots were probed with the ESAT-6 specific mAb, 76-8. In Figure 6.6a, the dot blot demonstrates a signal for all samples of CF, except MM grown in 7H9/OADC medium. The same negative result was true for MTB CF grown in 7H9 with the same supplement (data not shown). The intensity of the signal was similar for all three organisms. A second batch of MM CF (MM 2) was prepared and the organism was ZN-stained which confirmed the presence of acid fast bacilli. A Western blot was performed (Figure 6.6b). This demonstrated a band of the correct molecular

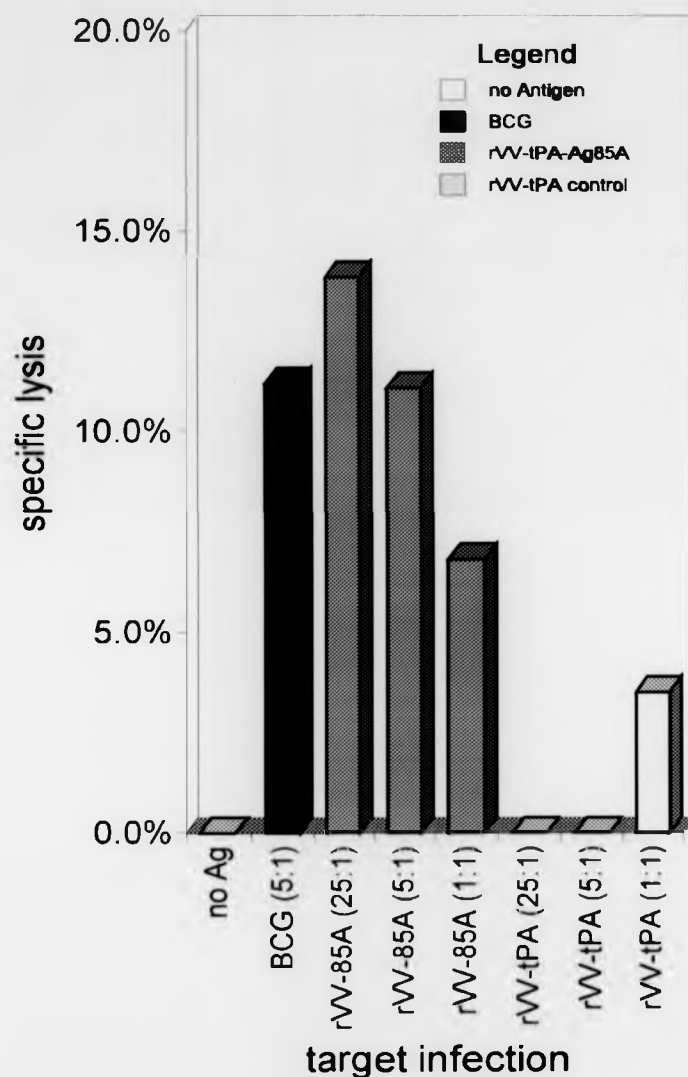


Figure 6.5 CTL assay using rVV-infected macrophage targets at a range of moi's PBMC from a healthy BCG vaccinee were restimulated with BCG to induce rVV-Ag85-specific CTL. Macrophage targets were infected rVV-tPA-85A (abbreviated to rVV-85A) or rVV-tPA lacking the gene insert, using three moi's (25:1, 5:1, 1:1). Additional targets were either mock infected or infected with BCG alone (moi 5:1) and a standard 6 hour ^{51}Cr release assay was performed.

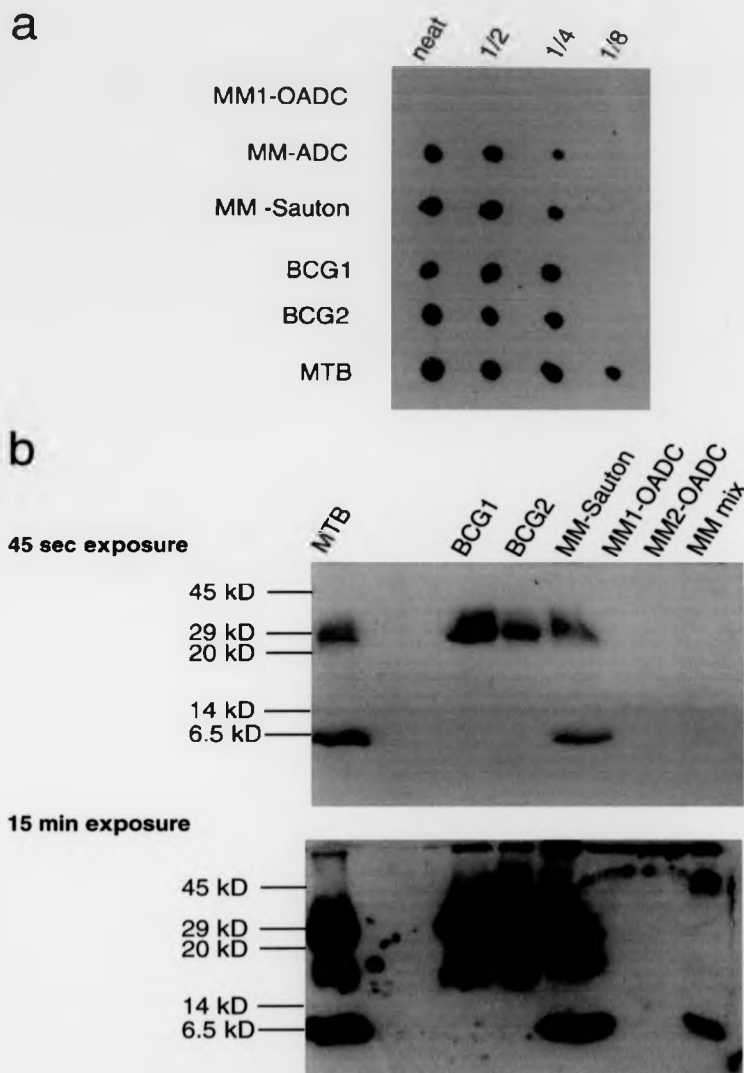


Figure 6.6 Immunoblots of MM, BCG and MTB CF probed for ESAT-6

A variety of CF preparations from 3 different mycobacteria grown in different media were subjected to dot and Western blot analysis using the ESAT-6-specific mAb, HYB76-8. The samples were derived from *M. marinum* (MM) grown in Sauton's medium (MM-Sauton), MM grown in 7H9 medium with OADC supplement (2 batches: MM1- & MM2-OADC); MM grown in 7H9 with ADC supplement (MM-ADC); BCG grown in 7H9/OADC (two batches: BCG1 & BCG2); and MTB grown in 7H9/OADC. **a)** A dot blot showing 1 µl aliquots of neat CF (1 mg/ml) and a series of twofold dilutions. **b)** Two exposures of the same Western blot showing ESAT-6 reactivity (6 kD bands) and additional 'cross-reactive' bands. The far right lane (MM mix) was loaded with an equal mix of MM1-OADC and MM-Sauton.

weight for MTB CF (Sauton's-derived) and MM CF (Sauton's-derived), but not for *M. marinum* grown in 7H9/OADC (MM 1 and MM 2). Furthermore, one of the OADC containing CF samples was able to inhibit the signal of MM CF (Sauton's-derived) when loaded as a 50% mix, as shown in the last lane (MM mix), although the signal did become apparent after a prolonged (15m) autoradiographic exposure (lower image). The Western blot also confirmed that the dot blot signal seen with BCG CF was not ESAT-6, but due to cross-reactivity with other secreted proteins including the 30-32 kDa proteins (Harboe et al. 1996). This experiment confirmed the finding of others, that MM expresses a protein with a molecular weight consistent with ESAT-6. Furthermore, using the ECL Western blotting system, growth of MM using OADC (but not ADC) supplement inhibited the signal. A similar pattern was observed with MTB CF. The key differences between the supplements are the presence of both oleic acid and catalase in OADC. Further experiments, not appropriate for this study, would be required to elucidate the mechanism of loss of signal.

6.2.2.2 Using CF-coated latex beads

One micron beads were coated with MTB CF (Copenhagen batch). The protocol entailed covalent linking of the CF proteins to 1 μ m carboxylated beads as described in Section 2.18.3.1. Coated beads were added, in three different amounts (40, 4 and 0.4 μ l), to a seven day macrophage culture. Following an overnight incubation, the cells were fixed with acetone and stained using the K92 polyclonal antibody, an HRP-antibody conjugate secondary antibody and DAB (Section 2.18.3.2). No specific signal for MTB CF was detected as compared with BSA-coated beads. However, of the three quantities tested, the lowest amount (0.4 μ l) provided ample uptake by all macrophages. Indeed, the macrophages incubated with 40 μ l were stuffed with beads and markedly damaged (data not shown).

Using the CTL ^{51}Cr release assay protocol, the lowest concentration of beads was selected to restimulate effector T cells and/or pulse macrophage target cells. Positive controls included live BCG, and negative controls included BSA-coated beads. These controls were used to pulse target cells and restimulate the effector cells. Microscopy of bead-restimulated PBMC suggested clonal expansion by day seven of the assay. However, in this single assay, the controls were unsuccessful and the results could not be interpreted.

6.2.3 CTL using a peptide approach

An alternative approach to using whole antigen or organisms to restimulate CTL is the use of peptides. The advantages and disadvantages of this are discussed in Section 3.3.2.2. This strategy was pursued by Lalvani and colleagues (1997) in Prof A. Hill's laboratory in Oxford. This group had a similar interest in both Ag85 proteins and ESAT-6 and a collaborative link was established, enabling the exchange of reagents. In particular, rVV-tPA-ESAT-6 was given to Prof Hill's group so they could assess the efficacy of peptide-induced CTL recognition of endogenously processed whole recombinant antigen.

In order to restimulate CTL, they scanned the sequences of ESAT-6 and Ag85 proteins for allele-specific peptide motifs for the HLA class I types -A2, -B7, -B8, -B35 and -B53. These alleles were selected on the grounds that they occurred with reasonable frequency in the study population. In all, 49 peptides were identified, synthesized and sorted into pools according to their HLA class I allele. The peptide pools were then used to restimulate PBMC derived from patients with active TB, treated TB patients and healthy controls. The *in vitro* expanded T cell populations were then used in two assays to assess function: (i) a highly sensitive ELISPOT technique for detecting IFN- γ from single cells (Miyahira et al. 1995); and (ii) a 5 hour ^{51}Cr release CTL assay.

The Oxford group identified two ESAT-6 CD8+ T cell epitopes from a panel of seven peptides. In contrast, no Ag85 CD8+ epitopes were identified from a possible 42 peptides. Whilst *in vitro* stimulation induced clonal expansion of putative anti-Ag85 CTL, the short term cell lines were all of the CD4+ T cell phenotype. Interestingly, some of these nonamer peptides elicited an IFN- γ response from uncultured CD4+ T cells (Lalvani A, Brookes R, Wilkinson RJ, Pasvol G, Hill AVS, unpublished data).

One of the ESAT-6 CD8+ peptides, ES12, was further characterised using a ES12-specific CD8+ clone. HLA class I restriction was formally demonstrated by comparing matched and mismatched EBV-transformed B cell lines (BCL), pre-pulsed with ES12 using the ELISPOT assay. In order to show that the ES12-specific clones could recognise endogenously-processed whole ESAT-6 protein, autologous BCL were infected with rVV-tPA-ESAT-6 or a control rVV lacking the ESAT-6 coding sequence. Using both the ELISPOT and CTL assay, the clones only recognised rVV-ESAT-6-infected BCL (Lalvani et al. 1997).

6.2.4 Murine immunisation using rVV-Ag85 and rVV-ESAT-6 constructs

rVV generated in this study have been used to immunise mice in laboratories in Brussels and Copenhagen. The key preliminary data come from the Brussels group and these are discussed below.

6.2.4.1 Spleen cell IL-2 and IFN- γ secretion following rVV-Ag85 immunisation

In Brussels, all the rVV-Ag85 constructs and a 'no antigen' control were used to immunise female C57BL/6 mice using 10^7 pfu given by the intravenous route. At 30 days, spleens were harvested from two mice per group. Spleen cells were cultured at 4×10^6 cells/ml in round-bottomed 96 well plates using 180 μ l of cells (7.2×10^5) plus 20 μ l of antigen (5 μ g/ml). The antigen consisted of the three Ag85 complex proteins (85A, 85B and 85C) purified from *M. bovis* BCG culture filtrate (De Bruyn et al. 1987). Supernatants from three wells were collected and pooled after 24h for an IL-2 assay and 72h for an IFN- γ assay. Cytokines were measured using two bioassays: (i) IL-2 by CTLL-2 cell proliferation; and (ii) IFN- γ by the CPE reduction assay of vesicular stomatitis virus on mouse L929 cells (Huygen et al. 1992). The IL-2 assay had a sensitivity of 10 pg/ml ($10\,000$ cpm \approx 150 pg/ml) and the IFN- γ assay had a sensitivity of 110 pg/ml ($\text{Log}_2 1 = 220$ pg/ml, $\text{Log}_2 2 = 440$ pg/ml etc).

Figure 6.7 shows the results of both IL-2 and IFN- γ secretion from mouse immune spleen cells in response to the recall antigen. The different rVV immunisation groups are represented along the X-axis and include all rVV-Ag85 constructs with and without the signal sequence. A clear pattern was apparent. Strong cytokine responses were induced from spleen cells of mice previously immunised with rVV-tPA-85A and -tPA-85B for both IFN- γ and IL-2. In contrast, weaker responses were obtained from the corresponding non-tPA constructs, rVV-85A and rVV-85B. However, standard deviations were too large to confirm a definite biological effect induced by the constructs lacking the signal sequence, except for IL-2 production in the rVV-85B immunised mice. The rVV-85C constructs failed to induce any detectable immune response using this system. Dr K. Huygen's group also measured antibody responses which showed a similar pattern, with higher titres for tPA than non-tPA Ag85A and 85B constructs (data not shown).

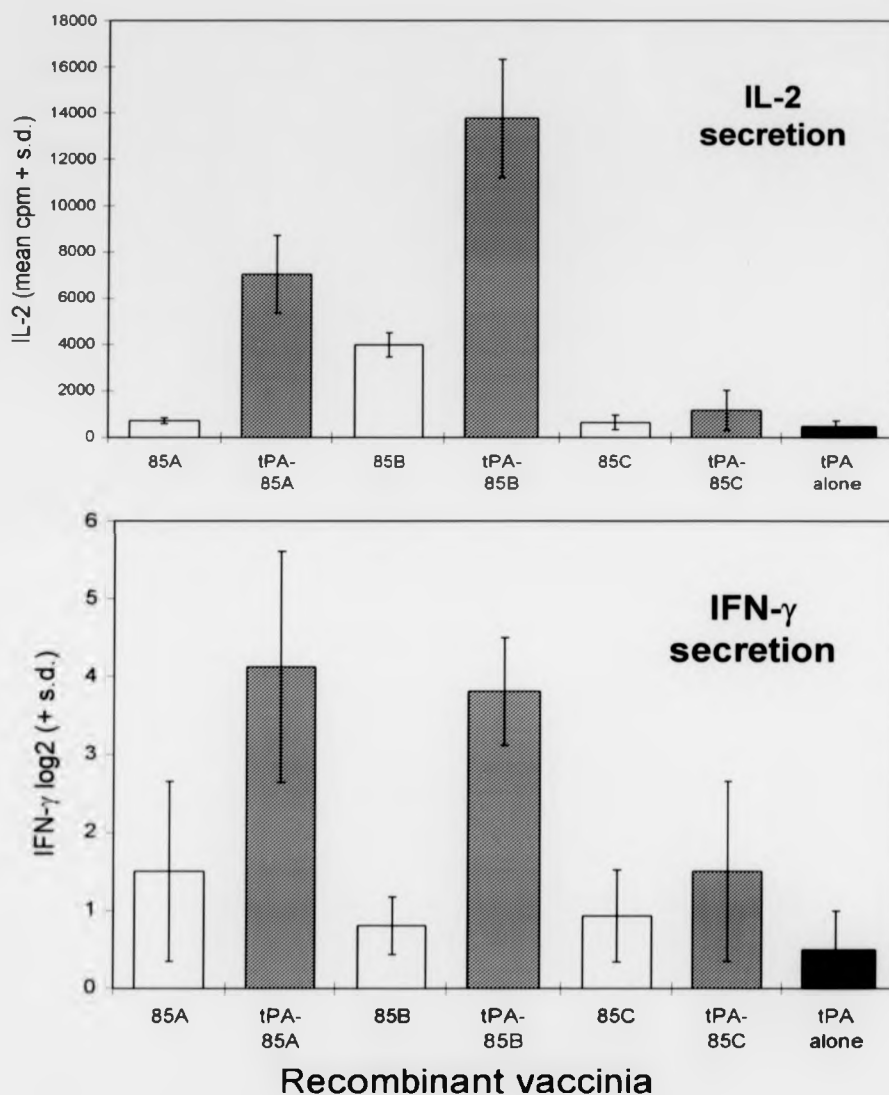


Figure 6.7 Cytokine secretion from spleen cells derived from rVV-immunised mice. IL-2 and IFN- γ secretion by immune spleen cells in response to Ag85 recall antigen. Each bar represents the mean cytokine bioassay readout (± 1 s.d.) from two B6 rVV-immunised mice, 30 days post infection. The IL-2 readout represents 150 pg/ml per 10 000 cpm (CTLL-2 proliferation). The IFN- γ readout represents 220 pg/ml for Log_2 (CPE reduction assay). Open bars rVV with no signal sequence, grey bars rVV with signal sequence.

6.2.4.2 rVV-ESAT-6 immunisation

Dr P. Andersen's laboratory in Copenhagen immunised mice using a different immunisation protocol. As an initial experiment, they selected a lower dose of rVV (10^6 pfu per mouse), given intravenously on two occasions, one month apart. Four groups of female C57BL/6 mice were compared: rVV-tPA-ESAT-6, rVV-tPA-85B, rVV-tPA and non-immunised mice. They failed to identify an immune response or protective efficacy using rVV-tPA-ESAT-6. rVV-tPA-85B showed raised IFN- γ responses but only a slight protective efficacy (0.3 log reduction in MTB CFU counts from lung and spleen organ homogenate as compared with the negative control). However, this was a single set of experiments in which there had been problems with high backgrounds from the cells from non-immunised control mice (Ms L. Brandt, personal communication). These experiments are being repeated using a higher immunisation dose of 10^7 pfu per mouse.

6.3 DISCUSSION

Data from three sets of experiments showed successful application of the rVV generated in this study. First, PBMC derived from healthy BCG vaccinees could be restimulated with live BCG to induce specific CTL recognition of rVV-tPA-Ag85A infected macrophage target cells. Second, PBMC derived from TB patients could be restimulated with an MHC class I allele-specific ESAT-6 peptide to induce a CD8+, MHC class I-restricted T cell clone with the capacity to produce IFN- γ and lyse rVV-tPA-ESAT-6 infected B cell lines. Third, a single intravenous immunisation of female C57BL/6 mice with rVV-tPA-85A and tPA-85B was able to induce IL-2 and IFN- γ cytokine production from spleen cells in response to purified Ag85 proteins. These data will be discussed in terms of their completeness, possible interpretation and the need for additional experiments.

6.3.1 BCG restimulation of CTL

One quarter of the CTL assays performed suggested that CTL from BCG-vaccinated healthy donors could recognise rVV-encoded Ag85 in a specific way. However, the data described in Section 6.2.1 represents only preliminary work using this approach. The time limitations of the study prevented further delineation. Additional data was required to both confirm the finding and identify the cell population(s) responsible. These issues are as follows.

6.3.1.1 *The need to control for non-specific lysis*

The initial assays, employing the positive BCG control only, showed a dose effect by varying the effector:target ratio (Figure 6.3). A different dose effect was demonstrated in CTL #4 (Figure 6.4) and CTL #8 (Figure 6.5), where the moi was varied for BCG and rVV respectively. Whilst this provides support for the target cell lysis being dependent on both the type and quantity of antigen, it is not conclusive. One might argue that these particular antigens (BCG and rVV-tPA-Ag85A) could render the target cells more prone to non-specific killing. This issue should be addressed by including effectors stimulated with an irrelevant antigen and including rVV-infected target cells with a construct expressing a larger protein than the tPA signal sequence alone construct e.g. rVV- β -gal or rVV-MOMP. If the HLA type of the subject were known, use of an appropriate peptide for PBMC restimulation and a rVV-flu construct for target infection would represent an effective control.

6.3.1.2 *Reproducibility of CTL*

Where specific CTL was seen, the assay should be repeated using PBMC from the same subject. Whilst CTL may change during the course of active disease such as patients undergoing treatment (analogous with a mouse model of infection (Orme, 1987)), one would expect that a healthy BCG vaccinee population, would have a similar CTL pre-cursor frequency from one point in time to another. Thus, the result should be reproducible.

6.3.1.3 *Effect of varying effector: target ratios*

Given the variability encountered in the results, with only 25% of assays yielding adequate control responses, further evidence is required to support that the data yielding a positive result represented a true biological effect rather than one that might have occurred by chance. For example, the data shown in Figure 6.4, if taken alone, could represent two apparently successful assays among many not displaying this trend. Demonstration of a dose effect using two or more effector:target ratios (depending on the number of lymphocytes available), would address this issue as provided in Figure 6.5.

6.3.1.4 *The role of CD4⁺ and CD8⁺ T cell subsets*

The restimulated PBMC should be defined in terms of T cell subsets. Work from others would suggest that a large proportion of activated T cells would be either CD4⁺ or CD8⁺ T cells (Turner and Dockrell, 1996). Purification of the restimulated T cells into CD4⁺ and

CD8+ T cell populations would permit assessment of the relative contribution made by each of these groups in the CTL assay. Once CD8+ CTL had been demonstrated, it would be appropriate to demonstrate that they were MHC class I-restricted, either by using blocking antibody or mismatching target cells. This approach had already been pursued in our laboratory by others using live and dead BCG and PPD for both T cell restimulation and target cell pulsing. These experiments had confirmed that these antigens induced cytotoxic CD8+ T cells, but had failed to prove that these CD8+ CTL were MHC class I restricted (Turner and Dockrell, 1996).

6.3.2 Peptide restimulation of CTL and MHC class I restriction

6.3.2.1 *The value of a defined CD8+ T cell ligand*

Proof that human CD8+ CTL are MHC class I restricted can be better addressed by developing T cell lines or clones which recognise known target cells. This was achieved by Prof A. Hill's group using peptide prediction to identify potential CD8+ T cell epitopes. The advantages of this system are twofold. The specific T cell ligand (peptide/HLA allele) is known, allowing molecularly defined manipulation of the system to show both peptide and MHC class I specificity. In addition, from a practical perspective, use of non-physiological APC's (EBV-transformed B cell lines) and T cell lines permits repeated assessment under a variety of assay conditions e.g. ELISPOT, CTL with varying E:T ratios and mismatching target cells.

The group in Oxford were able to demonstrate clearly that CD8+ MHC class I-restricted T cell lines could be raised using a predicted ESAT-6 peptide, ES12. These cells possessed IFN- γ secreting and CTL function. They recognised endogenously processed rVV-encoded ESAT-6. Furthermore, using the very sensitive ELISPOT assay, the peptide was able to induce IFN- γ production from unstimulated T cells taken straight from the TB patient from which the clone had been derived. The latter experiment showed that the pre-cursor frequency was sufficiently high to suggest biological relevance.

6.3.2.2 *The biological relevance of a peptide system?*

A key problem with this peptide approach is that it represents a self-selecting system. If these data were taken alone, it would be difficult to interpret the biological importance of the CD8+ T cells in their role in immunity against MTB. Because MHC class I peptides were chosen to restimulate T cells, with the addition of IL-7 and IL-2, using repeated

rounds of stimulation, there is a possibility of expanding T cell lines which may not be of biological relevance. IL-7 is known to facilitate the induction of primary CTL in PBMC derived from humans naive to the antigen (Plebanski et al. 1995). Furthermore, this study identified CD8+ CTL in only a small proportion of TB patients, despite the fact that high levels of T cell reactivity (proliferation and cytokine responses) had been identified using PBMC from TB patients in response to the whole ESAT-6 and Ag85 antigens (Huygen et al. 1988; Boesen et al. 1995). This disparity may reflect the limitations of testing for CTL reactivity using only a few single peptides. Lavani and colleagues (1997) screened only a few of over 1000 potential protein targets. However, the study represents the first time that human IFN- γ secreting CD8+ T cells, specific for an immunodominant MTB protein, have been shown to be MHC class I restricted.

It would be useful to develop a system where CD8+ CTL could be restimulated using whole antigen(s) or organism. This would increase the possibility of inducing a wider range of CTL which would be in proportion to the degree of antigen processing and presentation and independent of peptide prediction (Sadovnikova et al. 1994). This will be discussed in chapter 7.

6.3.3 Mouse immunisation

6.3.3.1 Cytokine responses from immune spleen cells

Dr K Huygen's group were able to demonstrate high levels of IFN- γ and IL-2 secretion from spleen cells derived from rVV-tPA-Ag85A and rVV-tPA-Ag85B immunised mice in response to purified Ag85 complex proteins. The cytokine secretion was either less or no better than background for mice immunised with constructs without the signal sequence (Figure 6.5). This trend reflected the pattern of expression seen using immunoblotting of Ag85 proteins (Figures 5.1 and 5.2) and a capture ELISA for ESAT-6 (Figure 5.7). It would be a mistake to interpret these data as suggesting that recombinant protein without the signal sequence is less immunogenic, as only one aspect of the immune response was assessed. It is likely that this difference reflects the preferential MHC class II processing of recombinant antigen within a vacuolar compartment and perhaps secreted in non-degraded form for further presentation by professional APCs. However, recombinant protein without the signal sequence may be more efficiently presented with MHC class I, analogous to signal sequence deletions of influenza nucleoprotein or haemagglutinin (Townsend et al. 1988). This hypothesis was not addressed in this study. In Prof A Hill's group, the rVV-tPA-

ESAT-6 construct was very efficiently recognised by the T cell clone, so it is unlikely that they would identify a difference between this construct and rVV-ESAT-6 alone.

6.3.3.2 *A potential poxvirus vaccine?*

From the perspective of a potential vaccine, the constructs with tPA would seem to be more promising. Firstly, the preliminary cytokine data suggested that they induced a definite Th1 response which was stronger than without tPA, an essential component of protective immunity (Orme et al. 1993). Furthermore, DNA vaccination data has suggested that the plasmids which included tPA were more immunogenic (Huygen et al. 1996).

However, numerous studies have been able to immunise mice and detect strong cytokine responses using different immunogens given with a variety of antigen carriers (Silva and Lowrie, 1994; Baumgart et al. 1996; Huygen et al. 1996; Lindblad et al. 1997). The key issue is whether the rVV can induce long-lived protective immunity in the available models, particularly in response to low dose aerosolised challenge with MTB. The laboratories in Brussels and Copenhagen are currently assessing protection, using MTB intravenous challenge (and also BCG challenge in Brussels). These data will give an early indication as to whether a poxvirus may represent a suitable vehicle for a future TB vaccine.

6.4 CONCLUSION

Functional application of rVV expressing Ag85 and ESAT-6 secreted proteins of MTB was demonstrated in three separate laboratories. In this laboratory, rVV-tPA-85A was used to infect human macrophage target cells in a ^{51}Cr release CTL assay. Preliminary data showed that these target cells were specifically lysed using BCG-restimulated whole PBMC from BCG vaccinees. From the laboratory of A Hill, Institute for Molecular Medicine, Oxford, an MHC class I restricted CD8⁺ T cell clone and short term T cell lines were raised from TB patients using a predicted peptide, ES12, from ESAT-6. These T cells were able to produce IFN- γ and lyse peptide-pulsed or rVV-tPA-ESAT-6 infected B cell line target cells. From the laboratory of K Huygen, Pasteur Institute, Brussels, rVV-tPA-85A and rVV-tPA-85B were able to immunise mice and produce IFN- γ and IL-2 responses following *in vitro* challenge of immune spleen cells with purified Ag85 proteins. Mouse protection studies, which are in progress, will show whether these rVV can induce protective immunity against BCG and MTB infection.

7 Final discussion

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7.1 IMPORTANCE OF THIS STUDY

Tuberculosis is a disease of superlatives. *Mycobacterium tuberculosis* causes more deaths annually than any other infectious agent. Globally, it is the major pathogen associated with HIV disease (Fine and Rodrigues, 1990; Bloom and Murray, 1992). The vaccine, BCG, has been given to more people than any other vaccine. Coverage now is approximately 85% of the world's population. Whilst BCG confers clear benefit against disseminated childhood tuberculosis, efficacy against the predominant adult pulmonary disease has varied widely in different clinical trials (Fine and Rodrigues, 1990). Curiously, protection induced by BCG appears to be better, the further one is from the equator (Fine and Rodrigues, 1990). Indeed, in large studies in India and Malawi, BCG was ineffective as a vaccine against pulmonary tuberculosis ("The Tuberculosis Prevention Trial", 1980; Ponnighaus et al. 1992). Given that tuberculosis trials are both costly and time consuming, there is a need to identify a short-term surrogate marker of potential efficacy; the current Mantoux or Heaf tests, based on skin test hypersensitivity, do not reflect protection (Fine et al. 1994). Alternative vaccine and diagnostic strategies are therefore urgently required.

A sub-unit vaccine approach is attractive in terms of both quality control and safety. However, in order to pursue a rational design strategy, it is necessary to know the protective antigens and the cellular components which are required for immunity (Malin and Young, 1996). An increasing body of evidence supports an immunodominant role for *M. tuberculosis* secreted proteins in protection (Andersen et al. 1991; Orme et al. 1992; Horwitz et al. 1995), particularly Antigen 85A (Andersen et al. 1992; Huygen et al. 1994; Huygen et al. 1996), 85B (Andersen et al. 1992; Horwitz et al. 1995) and ESAT-6 (Andersen and Heron, 1993; Sorensen et al. 1995). Secreted proteins have induced protective immunity in both mouse (Andersen, 1994) and guinea pig (Horwitz et al. 1995) models. In addition, DNA vaccination with an Ag85A-encoding construct has induced protection to the same order of magnitude as BCG (Huygen et al. 1996).

The cellular components of immunity include a key role for Th1-type CD4+ T cells and macrophages (Orme et al. 1993). However, other cell subsets may also be essential. Evidence in mouse gene knock-out models has implicated an additional requirement for class I-restricted T cell involvement (Flynn et al. 1992; Ladel et al. 1995). Direct evidence for murine CD8+ T CTL comes from immunisation of MHC class I congenic mice with BCG (Denis et al. 1997). CD8+ T cells have been shown to be cytolytic, but they have also been shown to release IFN- γ , which also plays a role in protection (Carter and Dutton, 1996). To dissect this problem, two groups studied the course of infection in perforin gene-knockout, and granzyme gene-knockout mice. They showed no evidence for the importance of these mediators of CD8+ T cell lytic activity (Cooper et al. 1997; Laochumroonvorapong et al. 1997). This suggests that cytokine secretion may be the major protective mediator of CD8+ T cells.

Mounting evidence exists for CD8+ T cell involvement in human immunity to tuberculosis. *In vitro* studies have identified mycobacterial-specific human CD8+ T cells (Rees et al. 1988). Moreover, CD8+ CTL from BCG vaccinees (Turner and Dockrell, 1996) and tuberculosis patients (Turner, unpublished results) can be generated by restimulating peripheral blood mononuclear cells with live BCG. These studies did not identify the MHC molecules responsible for antigen presentation. Therefore, whilst classical class I MHC restriction seems likely, alternative ligand recognition, such as in association with CD1, could not be excluded. In another study, CD8+ re-stimulation of CTL from TB patients has been achieved utilising a peptide approach. CD8+ T cell lines recognized both ESAT-6

peptide and endogenously processed recombinant ESAT-6 expressed in vaccinia, in a class I restricted manner (Lalvani et al. 1997). Further evidence suggestive of a role for CD8+ T cells comes from murine vaccination data using single mycobacterial antigen delivery systems, transfected tumour cells and DNA vaccination. Both systems permitted class I, as well as class II, antigen processing (Silva and Lowrie, 1994; Huygen et al. 1996; Tascon et al. 1996).

It is unclear how mycobacterial antigens enter the class I processing pathway. Possible explanations include escape of whole organisms into the cytosol (McDonough et al. 1993), regurgitation of processed antigen (Pfeifer et al. 1993), or egress of soluble proteins through pores within the membrane of the TB phagosome (Mazzaccaro et al. 1996). Evidence is accruing in favour of the latter phenomenon. Mazzaccaro and colleagues (1996) provide data to suggest that MTB releases mycobacterial proteins into the cytoplasm in a manner analogous to *Listeria* with TAP-dependent class I antigen processing. The same was true, to a lesser extent, with BCG. This lends support for the hypothesis that BCG may fail to elicit protection in some cases due to inadequate induction of a CD8+ T cell response. Overall, these studies suggest the need for an antigen delivery system capable of both class I and class II antigen processing for maximum protection. Such a delivery system would have utility, both as a potential vaccine carrier and also as a tool to specifically address the role of MHC class I-restricted T cell responses. One such carrier includes the poxvirus family.

Vaccinia, the archetypal poxvirus, is a cytoplasmic virus and, in recombinant form, it permits both class I and class II processing of antigen with good induction of CD4 and CD8 T cells (Tartaglia et al. 1990). From a vaccination perspective, attenuated poxviruses, such as modified vaccinia virus Ankara (MVA), are safe in the immunocompromised host but remain highly immunogenic (Tartaglia et al. 1990). These factors prompted us to make a panel of vaccinia recombinants (rVV) to express the immunodominant proteins of the Antigen 85 complex and ESAT-6. In order to enhance expression and improve immunogenicity, the signal sequence of tissue plasminogen activator (tPA) was included as an N-terminus fusion and these constructs were compared with those expressing the mature protein alone.

7.2 KEY FINDINGS

This study describes the construction of rVV expressing *M. tuberculosis* Ag85 and ESAT-6 proteins and their functional application, both as immunogens and tools to present processed antigen in the context of MHC class I. A marked enhancement of expression was noted using the heterologous eukaryotic signal sequence and, in the case of Ag85A and Ag85B, this was accompanied by enhanced *in vivo* cytokine responses to recall antigen (Huygen, unpublished results). The same feature was noted with rVV-ESAT-6 constructs where the quantity of recombinant protein was more than tenfold greater with tPA than without the signal sequence (Harboe et al. 1997). This enhanced secretion had been demonstrated previously in an *in vitro* HIV gp160 expression system (Chapman et al. 1991). Furthermore, Ag85A, also used in this study, had been expressed previously in a transfected cell line, both with and without tPA. The presence of tPA led to the secretion of the protein into the culture media (Huygen et al. 1996). In the same investigation, tPA was included in an Ag85A DNA construct for murine vaccination. This construct was able to confer protection in murine models to an order of magnitude similar to BCG, although there was no difference in protective efficacy between Ag85A constructs, both with and without tPA (Huygen and Content, unpublished results).

The present study was unable to show evidence of protein secretion for the rVV-tPA-Ag85A and -85B fusions. Perhaps protein secretion occurred, but at a level undetectable using immunoblotting. Alternatively, the signal sequence may have permitted translocation into a vacuolar compartment but been inadequate to permit trafficking to the cell surface for secretion into the extracellular environment. There is strong evidence for membrane translocation and post-translational modification of rVV-tPA-85B, as three glycoforms were identified. N-glycosylation was confirmed showing a reduction in molecular weight following N-glycosidase digestion of rVV-encoded tPA-85B but not 85B without tPA.

Ag85C expression was not identified for either of the rVV constructs, despite testing with a variety of antibodies. The available antibody probes were raised using native mycobacterial proteins and the possibility existed that these would fail to detect a heavily glycosylated eukaryotic cell-derived 85C protein. Therefore, sera from mice immunised with Ag85 DNA constructs were used in further immunoblots. Serum samples were able to provide specific signals for rVV-encoded Ag85A and Ag85B proteins but not Ag85C. It is not clear why this was the case, as plasmid sequencing data confirmed PCR fidelity and T7 coupled *in vitro*

transcription/translation of the same insert confirmed open reading frame integrity. Interestingly, using this *in vitro* cell free system, 85C products appeared particularly unstable as compared with products from the other three genes.

In terms of cytokine responses, rVV-tPA-Ag85A and rVV-tPA-85B appeared to be the most immunogenic of the constructs, as measured by IL-2 and IFN- γ responses following *in vitro* stimulation of immune splenocytes using purified Ag85 recall antigen (Huygen, unpublished results). This pattern paralleled the relative increase in expression when tPA was included in the constructs. The enhanced immunogenicity could represent a quantitative difference reflecting the level of protein expression. In addition, signal sequence-mediated translocation to a membrane compartment might also reduce degradation of recombinant antigen prior to uptake by professional antigen presenting cells.

In terms of a potential vehicle for TB vaccination, vaccinia holds the esteemed position of being the only vaccine to eradicate a global disease. In addition, it has been used as a vector for expressing numerous foreign genes of human, animal, bacterial and viral origin (Tartaglia et al. 1990). Animal vaccination has protected against a large number of infections including influenza, herpes simplex, hepatitis B and rabies (Brochier et al. 1990; Tartaglia et al. 1990). Several MTB and *M. leprae* rVV have been made (Lyons et al. 1990; Baumgart et al. 1996) and protection in a murine BCG model has been demonstrated (Baumgart et al. 1996). Wild type vaccinia recombinants could lead to an overwhelming disseminated viraemia in the immunocompromised host. However, an attenuated poxvirus could be used as a human vaccine vehicle. The highly host-range restricted poxvirus, MVA, represents an attractive candidate. It has a vaccination programme 'track record' for safety and immunogenicity and it is able to process antigen in the context of class I and induce CD8+ T cells as well as CD4+ T cells (Sutter et al. 1994). In a recombinant form, it is immunogenic (Sutter et al. 1994) and its large size will permit expression of multiple proteins including immunomodulatory molecules. In addition, a number of gene deletions have rendered it replication-defective in humans and given it a favourable safety and immunogenic profile (Blanchard et al. 1997). Such recombinant constructs could also be used to restimulate CTL, without the MHC limitations imposed by a peptide or transfected cell line approach.

This study has demonstrated high level expression of rVV-encoded Ag85A, Ag85B and ESAT-6 under a vaccinia early/late promoter with use of the tPA signal sequence. Following

rVV-Ag85 immunisation of mice, IFN- γ and IL-2 production from splenocytes was considerably greater for those animals vaccinated with the tPA constructs (Huygen, unpublished results). Further animal protection studies are under way. Poxvirus expression of MTB secreted proteins therefore represents an attractive choice towards the rational design of an effective tuberculosis vaccine.

7.3 FUTURE WORK

7.3.1 Outline

In order to investigate the potential of poxvirus constructs expressing *M. tuberculosis* proteins, a potential next step would be to construct a panel of recombinant modified vaccinia virus Ankara (rMVA) to express immunoprotective secreted proteins of *Mycobacterium tuberculosis* (Antigens 85A and 85B and ESAT-6). These could then be used in a murine model of tuberculosis to assess: (i) the protective efficacy of rMVA constructs compared with BCG, (ii) the 'booster' efficacy of rMVA constructs compared with BCG; and (iii) the potential correlates of protection such as cytotoxicity mediated by CTL and secretion of IFN- γ .

If rMVA constructs induced a significant degree of protection, then the system could be manipulated to address effects of: (i) different vaccination routes such as parenteral versus respiratory, (ii) multiple versus single gene insertions; (iii) the addition of rMVA-encoded cytokines such as IFN- γ , granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-12 (IL-12); and (iv) directing recombinant antigens to different cellular sites, such as to the cytoplasm or to the membrane or inducing cellular secretion.

It would also be interesting to compare the vaccine potential of rMVA with alternative delivery systems expressing the same antigens, such as DNA vaccination. This would be feasible for both Ag85 and ESAT-6, as protection studies using DNA vaccination have already been carried out. An additional advantage of rMVA would be its minimal lytic activity *in vitro*. rVV derived from wild-type vaccinia are too lytic to be used to restimulate low precursor frequency CTL, and so the use of rMVA would provide a better tool for the identification of biologically important CTL epitopes.

7.3.2 Initial phase

Development of first generation rMVA TB constructs

In order to develop first generation rMVA TB constructs, vaccinia transfer plasmids made in this study, could be utilized to construct rMVA-ESAT-6, -85A and -85B. These plasmids express *Eco-gpt*, permitting positive selection with MPA (Sutter et al. 1994). Previously, rMVA construction utilized one of the strain's deletion loci. However, homologous recombination using thymidine kinase flanking homology is possible (Dr T. Blanchard, personal communication), thus permitting the use of transfer plasmids designed for wt VV (WR strain). Recombinant TB genes would be placed under the control of the vaccinia early/late promoter, p7.5. PCR could be used to confirm recombinants as specific primers and reactions have been previously optimised. Expression would be confirmed by immunoblotting using the already available mAbs. rMVAs could also be designed to express a fusion of an Ag85 protein and ESAT-6 (Ag85-ESAT-6 fusion transfer plasmids could be constructed by sub-cloning from available constructs). Thus, a single construct could be used to vaccinate against two key immunoprotective antigens.

Mouse TB protection model

In order to test rMVA recombinants, the mouse TB protection model could be used. A potential initial screening protocol could include immunisation with 10^7 pfu intramuscularly of rMVA or 5×10^4 CFU sub-cutaneously of BCG (Glaxo strain) into 6-8 week old C57BL/6J female mice. A proportion of mice from each group could be challenged at 28 days using 5×10^4 CFU intravenously of MTB H37Rv strain and the protective efficacy measured as reduction in CFU culturing spleen and lung homogenates on Middlebrook 7H11 media. The remaining mice would provide material for immunological studies to assess CTL and cytokine production such as IFN- γ . These could be compared with protective efficacy data, in order to assist in the identification of 'immunological correlates of protection'. Such studies could utilise single cell suspensions of spleen cells harvested from rMVA-vaccinated mice 4 weeks post-immunisation and be used in two 'screening' T cell assays.

(1) *CTL assay* - cells would be re-stimulated with antigen and IL-2 (10 U/ml). rMVA would be used to infect target cells in a standard ^{51}Cr release assay. CD4+ and CD8+ T cell sub-populations could then be assessed.

(2) *IFN- γ assay* - cells would be stimulated with recall antigen (the corresponding native MTB antigen preparation) and supernatants harvested at appropriate time points. Other

supernatants could be stored for subsequent cytokine analysis (IL-4, IL-6, IL-10, TNF- α and GM-CSF) for favourable rMVA candidates.

7.3.3 Subsequent phase

Promising candidates - a similar or greater reduction in CFU count as compared with BCG

Subsequent experiments would assess: (1) vaccine dose, (2) route of vaccine administration (e.g. intranasal versus parenteral), (3) route of MTB challenge (aerosolised versus parenteral); (4) time of challenge increased from 28 to 70 days post immunisation (in order to avoid a non-specific anti-inflammatory effect induced following vaccination); (5) effects in alternative strains and species including outbred mice and guinea pigs; and (6) effect of priming mice with BCG, then comparing a 'booster' immunisation with rMVA as compared with BCG.

Second generation rMVA - rMVA-IFN- γ and rMVA-IL-12

Recent studies using DNA vaccination have shown that an ovalbumin-IL-12 cytokine fusion construct produced better IFN- γ and CTL responses (Maecker et al. 1997). Co-expression of recombinant antigens with cytokines (IFN- γ and IL-12) should skew the immune response in favour of a Th1 profile thought to be important in immunity to TB. Murine cytokine genes could be amplified using PCR from murine genomic template or plasmid (if available) and cloned under the control of a different promoter, such as the strong synthetic early/late dsP promoter (Sutter et al. 1994). The transfer plasmid would consist of a co-expression cassette containing cytokine and antigen genes, flanked by viral genome homology plus a selectable marker (*Eco-gpt* or β -gal).

Additional strategies could be employed to further enhance rMVA immunogenicity. For example, multiple genes could be expressed in a single construct and compared with the first generation constructs. Potential expression could include genes expressed under different promoters, or as a single open reading frame encoding a polyprotein. One such likely candidate would express Ag85B, ESAT-6 and hsp10. By this stage it is also likely that other immunoprotective antigens might have been identified. Therefore, alternative genes thought to encode immunoprotective antigens could be included, such as other secreted proteins, heat shock proteins or novel proteins emerging from the MTB genome mapping project.

It would also be interesting to compare immunogenicity of a model antigen expressed at different cellular sites (cytoplasmic, membrane and secreted). Transfer plasmids and wt rVV were prepared in this study for both cytoplasmic and secreted expression (with and without the tPA signal sequence). Membrane expression would entail construction of a fusion protein with antigen expression at the extracellular domain of a transmembrane protein.

7.3.4 Potential long-term prospects

Should rMVA-TB constructs appear significantly immunogenic in the small animal models, candidate vaccine development could include: (i) re-construction with human cytokine genes (for cytokine-encoding rMVA); (ii) sub-human primate challenge (with the possibility of collaboration such as that in the Philippine facility (Walsh et al. 1996); (iii) re-construction of successful rMVA up to GMP standards and (iv) phase I/II studies.

7.4 CONCLUSION

This study has demonstrated high level expression of rVV-encoded Ag85A, Ag85B and ESAT-6 under a vaccinia early/late promoter with use of the tPA signal sequence. In CTL experiments, rVV-tPA-Ag85A-infected macrophage target cells could be lysed specifically by BCG restimulated human CTL derived from healthy BCG vaccinees. In addition, rVV-tPA-ESAT-6 represented a useful tool for the identification of human MHC class I-restricted, cytotoxic, IFN- γ -producing, CD8⁺ T cells derived from patients with tuberculosis. Following rVV-Ag85A and -Ag85B immunisation of mice, IFN- γ and IL-2 production from immune splenocytes was considerably greater for those animals vaccinated with the tPA constructs than those constructs lacking this signal sequence. Poxvirus expression of MTB secreted proteins therefore represents an attractive choice towards the rational design of an effective tuberculosis vaccine.

However, formidable problems will be faced in moving any vaccine from the laboratory into clinical trials. Whilst immunogenicity in animal models provides encouragement, it cannot be taken as a measure of protection in man. Seventy years of experience with BCG has shown the difficulty of evaluating a vaccine against tuberculosis. Development of an improved TB vaccine presents a daunting task in the face of our limited understanding of the organism's virulence and the host's immune response. Given the superlative impact of tuberculosis, we cannot avoid this task. In fact, we require superlative effort.

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Appendix A

	weight (g)
glucose	2
bovine serum albumin (BSA)	5
sodium chloride	0.85
water	100 ml final vol.

Table A.1 ADC supplement

Sauton's medium	weight (g)	procedure	Supplement
K ₂ HPO ₄	0.5	Dissolve in 600 ml of distilled water and adjust pH to 7.4 with NaOH and then autoclaved	28 g glycerol dissolved in 400 ml of distilled water and autoclaved. Added to the Sauton base just prior to use.
MgSO ₄ ·7H ₂ O	0.5		
citric acid	2		
L-asparagine	4		
(Ferric)FeNH ₄ citrate	0.05		

Table A.2 Sauton's media

Reagent	Amount (g) to be dissolved in 10 ml	Solvent	Stock concentration	Enzymes inhibited
EDTA	2.92 g	water	1 M	metalloproteases
EGTA	3.80 g	water	1 M	metalloproteases
NEM	1.25 g	water	1 M	thiol proteases
Pepstatin	6.85 mg	ethanol	1 mM	aspartyl proteases
PMSF	581 mg	ethanol	0.33 M	serine proteases
TPCK	352 mg	ethanol	0.1 M	serine, thiol proteases

Table A.3 Reagents used in protease inhibitor mix

EDTA=ethylene diamine tetraacetic acid, disodium salt; EGTA=ethylene glycol bis (2-amino ethyl ether) - N, N, N', N'- tetraacetic acid; NEM=N-ethylmaleimide; PMSF=phenyl methyl sulphonyl fluoride; TPCK=N-tosylamide-L-phenylalanine chloromethyl ketone. *=kept at pH 8.0 to maintain in solution.

Errata

- p12 DAPI - 'cell nuclei' should read 'DNA'
- p12 dNTPs - 'deoxynucleotide' should read 'deoxynucleoside'
- p13 FCS - 'fetal' should read 'foetal'
- p13 λ - 'lamda' should read 'lambda'
- p18 line 28 - 'unequal-equal' should read 'unequal'
- p23-24 bottom paragraph p23/top paragraph p24 - should read 'Initial work suggested that the terminal arabinose side-chains of *Mycobacteria* spp. could lead to a difference in strain virulence. This was based on data suggesting that the virulent MTB Erdman strain contained a relatively inert LAM with mannose-capping (ManLAM). In contrast, the less virulent H37Ra strain contained LAM lacking this mannose-capping (Ara LAM). This latter form of LAM was thought to more readily induce inflammatory cytokines, such as TNF- α , permitting more rapid clearance of the organism. However, subsequent data showed that LAM from the vaccine strain, *M. bovis* BCG, was also mannose-capped (Prinzis et al. 1993).
- p26 line 15 - 'units' should read 'unit'
- p27 line 15 - 'CD-1' should read 'CD1'
- p31 line 11 - 'was' should read 'were'
- p34 line 5 - 'by (Orme' should read 'by Orme'
- p35 line 25 - 'De et al' should read 'De Bruyn et al'
- p37 line 3 - 'ESAT6' should read 'ESAT-6',
- p37 line 17 - 'this protein' should read 'the ESAT-6 protein'
- p38 line 12 - omit '(Horwitz et al. 1995)'
- p41 line 7 - '(Smith. 1993)' should read '(Lane et al, 1969)'
- p42 line 5 - 'to grow in mammalian cell lines' should read 'to grow in many mammalian cell lines'
- p42 line 15. The sentence beginning 'Both IFN receptors . ' should read 'Both IFN receptors down-regulate immune responsiveness and the IFN- α/β R is associated . '
- p48 line 6 - 'EcoRI' should read 'EcoRI'
- p51 line 10 - insert comma after 'ESAT-6'
- p55 line 3 of bottom paragraph - omit 'required'
- p59 Table 2.2, row 9 - 'PRTK4294' should read 'PRTK429R'
- p60 line 11 - 'deoxynucleotide' should read 'deoxynucleoside'
- p60 second line from bottom - after '10 mCi/ml' should follow '(>1000 Ci/mmol)';
- p60 bottom line - after '15 mCi/ml' should follow '(>1000 Ci/mmol)'
- p61 line 1 - after '1-5 μ Ci/ml' should follow '(200-900 Ci/g Cr)'
- p61 line 20 - 'lamda' should read 'lambda'

- p71 line 10 - 'glutamax II' should read 'Glutamax I'
- p79 line 15 - '50 ml' should read '50 μ l'
- p80 line 6 - should read 'MgCl₂'
- p84 line 15 - 'protein gel electrophoresis' should read 'polyacrylamide gel electrophoresis'
- p87 line 15 - 'Photographed' should read 'Photography'
- p100 line 7 - '4 examples' should read '3 examples'
- p106 line 24 - '(lowest dilution)' should read '(3 μ g lane)'
- p112 bottom line - the reference 'Mackett, 1995' should be replaced with 'Yuen and Moss, 1987'.
- p113 top line - the first sentence should be replaced with 'The details concerning the 7.5K promoter have been identified previously (Cochran et al. 1985), including the transcriptional initiation site.'
- p117 bottom line - 'Tamron' should read 'Tamarron'
- p118 line third from bottom - replace 'Mackett, 1995' reference with 'Elroy-Stein et al. 1989'
- p124 line 7 - 'Figure 4.2c' should read 'Figure 4.2bii'
- p136 line 14 - '#12, #15, #17 and #20' should read '#12, #16, #18 and #22'
- p139 line 7 - '23/24' should read '23/26'
- p139 line 16 - 'e(i) and f' should read 'e and f(i)'
- p149 line 1 - omit second 'was'
- p165 line 17 - 'lane 8' should read 'lane 6'
- p167 line 13 - '(where Xaa is any residue)' should read '(where Xaa is any residue except proline)'
- p176 line 22 - 'rVV-Ag85,' should read 'rVV-Ag85A,'
- p189 line 5 - should read 'IFN- γ '
- p191 second line from bottom - 'was' should read 'were'
- p209 should include the reference, 'Cochran MA, Puckett C and Moss B (1985) In vitro mutagenesis of the promoter region for a vaccinia virus gene: evidence for tandem early and late regulatory signals. *J. Virol.* **54**, 30-37.'
- p214 should include the reference, 'Lane JM, Ruben FL, Neff JM and Millar JD (1969) Complications of smallpox vaccination, 1968. National Surveillance in the United States. *N. Engl. J. Med.* **281**, 1201-1208.'
- p220 should include the reference, 'Prinzis S, Chatterjee D and Brennan PJ (1993) Structure and antigenicity of lipoarabinomannan from Mycobacterium bovis BCG. *J. Gen. Microbiol.* **139**, 2649-2658.'
- p223 omit second Townsend et al. reference and change date on the first of these from '1988a' to '1988'
- p224 should include the reference, 'Yuen L and Moss B (1987) Oligonucleotide sequence signalling transcriptional termination of vaccinia virus early genes. *Proc. Natl. Acad. Sci. U. S. A.* **84**, 6417-6421.'



B-Cell Epitopes and Quantification of the ESAT-6 Protein of *Mycobacterium tuberculosis*

MORTEN HARBOE,^{1*} ADAM S. MALIN,² HAZEL S. DOCKRELL,² HARALD GOTTEN WIKER,¹
GUNNI ULVUND,¹ ARNE HOLM,³ MIKALA CLOK JØRGENSEN,⁴
AND PETER ANDERSEN⁵

Institute of Immunology and Rheumatology, University of Oslo, N-0172 Oslo, Norway¹; Department of Clinical Sciences, London School of Hygiene and Tropical Medicine, London, United Kingdom²; and Chemical Institute, Royal Veterinary and Agricultural University,³ and Clinical Biochemistry Department⁴ and TB Research Unit,⁵ Statens Seruminstitut, Copenhagen, Denmark

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ESAT-6 is an important T-cell antigen recognized by protective T cells in animal models of infection with *Mycobacterium tuberculosis*. In an enzyme-linked immunosorbent assay (ELISA) with overlapping peptides spanning the sequence of ESAT-6, monoclonal antibody HYB76-8 reacted with two peptides in the N-terminal region of the molecule. Assays with synthetic truncated peptides allowed a precise mapping of the epitope to the residues EQQWNFAGIEAAA at positions 3 to 15. Hydrophilicity plots revealed one hydrophilic area at the N terminus and two additional areas further along the polypeptide chain. Antipeptide antibodies were generated by immunization with synthetic 8-mer peptides corresponding to these two regions coupled to keyhole limpet hemocyanin. Prolonged immunization with a 23-mer peptide (positions 40 to 62) resulted in the formation of antibodies reacting with the peptide as well as native ESAT-6. A double-antibody ELISA was then developed with monoclonal antibody HYB76-8 as a capture antibody, antigen for testing in the second layer, and antipeptide antibody in the third layer. The assay was suitable for quantification of ESAT-6 in *M. tuberculosis* antigen preparations, showing no reactivity with *M. bovis* BCG Tokyo culture fluid, used as a negative control, or with MPT64 or antigen 85B, previously shown to cross-react with HYB76-8. This capture ELISA permitted the identification of ESAT-6 expression from vaccinia virus constructs containing the *esat-6* gene; this expression could not be identified by standard immunoblotting.

A key question in the development of new vaccines against tuberculosis is whether particular antigens of *Mycobacterium tuberculosis* are of major importance in the development of protective immunity.

Various techniques are used for the identification of protective antigens. In particular, we have studied the specificity of T-cell responses in a mouse model of memory immunity after infection with *M. tuberculosis* (1, 5). This system involves *M. tuberculosis* infection of C57BL/6J mice for 1 month, treatment with isoniazid and rifampin to clear the infection, and then resting for a longer period prior to reinfection. Following *M. tuberculosis* reinfection, these memory immune mice develop a rapid and intense T-cell response which controls the infection. By testing individual fractions of a short-term culture filtrate (ST-CF) enriched in proteins actively secreted by *M. tuberculosis* (4), the protective T cells were found to exhibit a very restricted specificity. Two fractions (3 to 10 and 25 to 31 kDa) were strongly recognized and induced both marked proliferative responses and high levels of gamma interferon (IFN- γ) release (5).

In the fraction containing 25- to 31-kDa proteins, the components of the antigen 85 complex are major constituents (34, 37). Among these, 85B has been demonstrated to induce proliferation and high levels of IFN- γ release in T-cell cultures from memory immune mice (1, 2). The 85A protein has previously been shown to induce IFN- γ production in cultures of

spleen cells from mice recently infected with *M. tuberculosis* (9). In agreement with this finding, antigen 85A has recently been shown to be protective in DNA vaccination (17).

In the second fraction, containing proteins ranging in molecular mass from 3 to 10 kDa, a protein with an apparent molecular mass of 6 kDa designated ESAT-6 (6-kDa early secretory antigenic target) was shown to possess the major activity (1). Overlapping peptides spanning the sequence of ESAT-6 have been used to map two T-cell epitopes on this molecule in mice. One epitope, recognized in the context of H-2^d, was located in the N-terminal part of the molecule; the other epitope, recognized in the context of H-2^m, covered amino acids 51 to 60 (7). The *esat-6* gene, which lacks a signal sequence (1, 30), is present in *M. tuberculosis* and virulent *Mycobacterium bovis* but absent in the *M. bovis* BCG vaccine strain (16).

One possible avenue toward improved vaccines against tuberculosis would therefore be recombinant live vaccine carriers such as BCG or attenuated vaccinia virus expressing ESAT-6 (13, 25). Specific quantification of ESAT-6 expression in sonicates and culture fluids of recombinant microorganisms in which the *esat-6* gene has been introduced is essential as part of the selection of candidate recombinant microorganisms for further vaccination experiments. The purpose of the present work was to characterize B-cell epitopes on the ESAT-6 molecule as a basis for the development of an enzyme-linked immunosorbent assay (ELISA) for its quantification.

MATERIALS AND METHODS

Bacterial cultures and antigen preparations. *M. bovis* BCG substrain Danish 1331 was obtained from Statens Seruminstitut, Copenhagen, Denmark, and

* Corresponding author. Mailing address: Institute of Immunology and Rheumatology, University of Oslo, Fr. Øyams gate 1, N-0172 Oslo, Norway. Phone: 47 22 03 31 70. Fax: 47 22 20 72 87. E-mail: morten.harboe@iuhmed.uio.no.

substrain Tokyo 172 was obtained from the National Institute of Health, Tokyo, Japan, and grown on Sauton medium. ST-CF, enriched in proteins actively secreted by *M. tuberculosis* H37Rv, was produced as described previously (3). Culture fluids, 3 to 5 weeks old from stationary cultures of *M. tuberculosis* H37Rv containing secreted proteins and only small amounts of cytosolic proteins released by bacterial lysis were prepared as described previously (27, 35).

Clinical isolates of *M. tuberculosis* were identified by standard diagnostic methods at the Mycobacteria Department, Statens Seruminstitut, grown on Ogawa slants or Löwenstein-Jensen medium, and then transferred to liquid Sauton medium for further cultivation.

Isolation of proteins. Proteins were isolated as described in detail previously, and then tested for homogeneity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), protein staining, and immunological techniques. MPT59 (85B) and MPT64 were purified from *M. tuberculosis* culture filtrate (27). The term MPB was introduced by Nagai et al. (26) for the designation of a protein purified from *M. bovis* BCG, with a number denoting the relative mobility in PAGE (7.7% polyacrylamide) at a running pH of 9.5. The corresponding term MPT is used to denote a protein isolated from *M. tuberculosis* H37Rv, purified from 3- to 5-week-old culture filtrate of *M. tuberculosis* H37Rv (30).

Preparation and purification of recombinant ESAT-6. A synthetic DNA sequence (5'-G CGA CAT CAC CAT CAC CAT CAC CAT CAC ATC GAG GGC A3' plus protruding 5' GATC overhangs), coding for a stretch of eight His residues followed by a factor Xa cleavage site, was inserted in the unique *Bgl*II recognition site of the expression plasmid pGH433 (31). This generated plasmid pMCT6, in which the *Bgl*II site downstream of the insert was maintained. The DNA sequence coding for the full-length ESAT-6 protein was PCR amplified from cloned *M. tuberculosis* genomic DNA with the primers 5'-GAAGATCTTA TGACAGAGCAGCAGTGG (nucleotides 1 to 18 of the coding sequence with a *Bgl*II site added upstream) and 5'-CCGCCATGGTAAACACGAGAAAGG GCG (nucleotides 67 to 84 after the stop codon, with a *Nco*I site added downstream) and inserted between the *Bgl*II and *Nco*I sites of pMCT6. The recombinant protein was produced in *Escherichia coli* XLI blue and purified by metal ion affinity chromatography on a Ni²⁺ column essentially as described previously (32) but with phosphate buffers containing 8 M urea, which was removed after the purification.

Hydrophobicity plots. Hydrophobicity plots were prepared by the method of Kyte and Doolittle (21).

Synthesis of ESAT-6 peptides and generation of antibodies. Monoclonal antibody (MAb) HYB76-8, reacting with ESAT-6, was obtained from purified protein derivative-immunized CF1 \times BALB/c F₁ mice (20). The MAb (batch 05C8.025B3) was purified by affinity chromatography on protein A-Sepharose CL-4B as specified by the manufacturer (Pharmacia Biotech, Uppsala, Sweden).

Eight overlapping 20- to 24-mer peptides, covering the amino acid sequence of ESAT-6 and a series of truncated peptides in the residue 1 to 20 region from the N-terminal end were synthesized as described in detail previously (7). To produce antibodies to peptides from regions within the ESAT-6 molecule presumed to contain B-cell epitopes, two 9-mer peptides (DEGKQSLTK, positions 30 to 38 [p30-38], and YOGVQKWD, positions 51 to 59 [p51-59]) were obtained from MedProbe A/S, Oslo, Norway, as peptide amides, blocking the C-terminal carboxyl group of the peptide to more closely mimic the charge environment of the native protein. A cysteine residue was added at the N terminus, using a part of the peptide for coupling to keyhole limpet hemocyanin (KLH) as the carrier protein for immunization. The heterobifunctional reagent *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) was used for coupling by a two-step procedure, first activating the carrier and then coupling the peptide (12, 19, 22). The rabbits were immunized by repeated subcutaneous injections of peptide coupled to KLH in an emulsion with incomplete Freund's adjuvant at multiple sites by standard procedures (14). The same technique was used to generate antibodies against the 23-mer peptide AAAGGSGSEAYOGVQKWDATA (p40-62). The rabbits were bled on the day before the first immunization, immediately before subsequent immunizations, or separately as indicated.

To obtain anti-p30-38 and anti-p51-59, 180 μ g of coupled protein was used to immunize each rabbit on day 1 and 90 μ g was used for each of the three subsequent immunizations 3, 5, and 9 weeks later. The rabbits were bled on day 1 and then 3, 7, 9, and 11 weeks later.

To obtain anti-p40-62, 830 μ g of protein was used to immunize each rabbit on day 1 and then 2, 9, and 13 weeks later. These animals were bled on day 1 and then 3, 9, 11, 13, 15, and 16 weeks later.

Polyvalent rabbit anti-BCG immunoglobulin was kindly provided by DAKO Immunoglobulins, Copenhagen, Denmark. Generation of polyvalent anti-*M. tuberculosis* culture fluid and monospecific polyclonal rabbit antibodies to isolated mycobacterial proteins has been described previously (35).

SDS-PAGE with immunoblotting. SDS-PAGE was performed with the Pharmacia system for horizontal electrophoresis in a Multifit II electrophoresis unit 2117 (Pharmacia LKB Bioteknology AB, Uppsala, Sweden) with precast polyacrylamide gels, ExcelGel SDS gradient 8-18 (Pharmacia). Samples (10 μ l) of the various culture filtrates containing 10 μ g/ml total protein were applied in each lane. Scanning Western blotting was performed with the model 2117-250 Nova-blot electrophoretic transfer kit (LKB, Bromma, Sweden) onto 0.2- μ m-pore-size nitrocellulose membranes (Schleicher and Schüll, Dassel, Germany). After electrophoretic transfer, antigen bands were detected with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit immunoglobulin (Amersham Interna-

tional plc, Little Chalfont, United Kingdom), with diaminobenzidine in 0.1 M sodium acetate buffer (pH 4.0) as the substrate.

ELISA technology. To test for antibody reactivity with synthetic peptides, the plates were coated with individual peptides by applying 1 μ g of peptide per well in 100 μ l of phosphate-buffered saline (PBS) (pH 7.4), blocking with 5 mg of bovine serum albumin per ml in PBS. For reactivity with MAb HYB76-8, detection was carried out with HRP-labelled sheep anti-mouse immunoglobulin (Amersham); for reactivity with polyclonal rabbit anti-peptide antibodies, detection was carried out with HRP-labelled donkey anti-rabbit immunoglobulin (Amersham).

Based on initial titer determination experiments outlined below, a double-antibody ELISA was set up according to the principle described in detail previously (16, 36). Briefly, Immulonate MaxiSorp 96-well plates (no. 442404; NUNC, Roskilde, Denmark) were coated with 100 μ l of purified MAb HYB76-8 (500 μ g/ml) diluted 1:200. Blocking was carried out with 5 mg of bovine serum albumin per ml in PBS. The second layer contained serial twofold dilutions of the antigen to be tested (culture fluids, sonicates, or purified proteins) in the concentrations indicated. Dilutions of culture fluids of *M. tuberculosis* H37Rv and BCG Tokyo were included as positive and negative controls, respectively. The third layer contained polyclonal rabbit anti-p40-62 peptide at 1:200. The indicator system consisted of HRP-conjugated donkey anti-rabbit immunoglobulin (Amersham) at 1:1,000. The substrate was 2,2'-azino-di-[3-ethylbenzothiazole-sulfonate] (16a) (ABTS). The samples were washed four times between each step with PBS containing 0.1% Tween 20. All reaction mixtures were set up in triplicate, and the median values were used for recording and calculations. The results were read on an MR 7000 ELISA reader (Dynatech Laboratories Inc., Chantilly, Va.).

rVV expressing ESAT-6. Two recombinant vaccinia virus (rVV)-ESAT-6 sequences were constructed with the following foreign coding sequences: (i) *esat-6* alone or (ii) *esat-6* fused downstream from a heterologous eukaryotic signal sequence, *tpa*, belonging to tissue plasminogen activator (tPA) (28). These were inserted into the nonessential thymidine kinase locus of wild-type VV (strain WR) with the transfer plasmids p1108 and pSC11. These transfer plasmids included a cloning site for foreign sequence insertion under the vaccinia early/late p7.5 promoter, flanking viral thymidine kinase sequence which permits homologous recombination and a selectable marker under a second VV promoter, and the *E. coli* genes, *gpt* for p1108 and *lacZ* for pSC11 (23). For rVV-tPA-ESAT-6, p1108-tPA was constructed by cloning synthetic sense and antisense oligonucleotide strands (MWG Biotech, Ebersberg, Germany) encoding the 21-amino-acid leader sequence of tPA and a multiple-cloning site containing *Nhe*I, *Bam*HI, *Sma*I, and *Eco*RI (24). An *esat-6* coding sequence was inserted into both p1108 and p1108-tPA on a *Bam*HI-*Eco*RI PCR-generated fragment with restriction sites incorporated within the primers. Following confirmation of plasmid sequence fidelity, homologous recombination into VV was carried out as described elsewhere (23). Briefly, a near-confluent monolayer of TK-143 cells was infected with wild-type VV at a multiplicity of infection of 1.10, followed 1 h later by transfection with p1108-ESAT-6 or p1108-tPA-ESAT-6 with the cationic lipid transfection reagent *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate (DOTAP; Boehringer, Mannheim, Germany). Viral recombinants were selected with mycophenolic acid (23), and three rounds of plaque purification were performed. rVV-ESAT-6 and rVV-tPA-ESAT-6 were verified with PCR primers both flanking the coding region and within it (*tpa* and *esat-6*). A similar procedure was used with the pSC11 transfer plasmid, but in this case, recombinants were color selected with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal; Sigma Chemical Co., St. Louis, Mo.). Protein cell lysates were obtained from rVV-infected TK-143 cells (multiplicity of infection, 30:1) 18 h postinfection. The cells were lysed in 1 ml of 1% Nonidet-P-40 in 50 mM Tris (pH 8.0)-150 mM NaCl, representing 8×10^6 cells/ml. The cell lysates were assayed for their protein concentration with the bicinchoninic acid protein assay reagent (Pierce Chemical Co., Rockford, Ill.) as specified by the manufacturer and stored at -70°C prior to use.

Southern blotting, RT-PCR, and Western blotting of rVV-ESAT-6. Southern blotting of rVV-ESAT-6 genomic DNA was performed with a digoxigenin-labelled whole-gene probe. Cell monolayers were infected with rVV-ESAT-6 and rVV- β -galactosidase (negative control), and genomic DNA was extracted by standard methods. Genomic and plasmid (pSC11-ESAT-6 and pSC11 alone) DNA were digested with *Eco*RI, resulting in a predicted 700-bp fragment incorporating *esat-6*. These digests were subjected to standard Southern blotting, and the signal was detected by a chemiluminescence method (Boehringer Mannheim) with an anti-digoxigenin alkaline phosphate-conjugate antibody, Lumigen purified protein derivative substrate, and a digoxigenin-UTP-labelled *esat-6* whole-gene probe as specified by the manufacturer.

Reverse transcription-PCR (RT-PCR) was performed for recombinant *esat-6* transcripts. mRNA was purified from infected cell lysates and reverse transcribed with SuperScript (Life Technologies, Paisley, United Kingdom). *esat-6*-specific PCR primers were used to amplify a predicted 300-bp product. Given that the mRNA preparation would be contaminated with viral genomic DNA (VV is a cytoplasmic virus), the samples were treated with DNase (Life Technologies) before the RT step. Relevant controls included no RT step (both with and without DNase treatment), no DNase treatment, a plasmid incorporating *esat-6* as a positive control, and cDNA from rVV lacking *esat-6* as a negative control.

Western blotting was performed on rVV-infected cell lysates. Samples were run on SDS-PAGE under both reducing and nonreducing conditions and with both large- and small-scale gel systems. Following protein transfer, the nitrocel-

Peptide	Sequence	Reactivity with HYB76-8
P1 1-20	MTEQQWNFAGIEAAASAIQG	+++
P2 12-35	EAASAIQGNVTISIELLDEKQS	+
P3 22-45	VTSIELLDEKQSLTKLAAAGG	-
P4 32-55	GKQSLTKLAAAGGSGSEAYQGVQ	-
P5 42-65	ANGGSGSEAYQGVQKMDATATEL	-
P6 52-75	QGVQKMDATATELNNALQNLART	-
P7 62-85	ATELNNALQNLARTISEAGQAMAS	-
P8 72-95	LARTISEAGQAMASTEIGNVTGMPA	-

+++ O.D. in ELISA >0.900
 + O.D. 0.100-0.200
 - O.D. <0.100

FIG. 1. Reactivity of overlapping synthetic peptides derived from the sequence of ESAT-6 in ELISA.

lucose membrane was probed with HYB76-8 followed by HRP-conjugated secondary antibody (P260, Dako). The blotting procedure and signal detection were performed as specified for the enhanced chemiluminescence Western blotting detection system (Amersham).

RESULTS

Reactivity of MAb HYB76-8 with ESAT-6. MAb HYB76-8 defines the ESAT-6 antigen (1, 16, 30). To precisely locate the epitope recognized by HYB76-8, a series of overlapping peptides covering the sequence was tested in an ELISA. Figure 1 shows the reactivity of eight synthetic 20- to 24-mer peptides on the solid phase with MAb HYB76-8 diluted 1:100. Reactivity is shown by +, ++, +, and - signs as indicators of the signal strength based on the optical densities (ODs) obtained on an ELISA plate in which the recorded peptides were tested simultaneously. Strong reactivity was observed with the peptide corresponding to residues 1 to 20 from the N-terminal end. Weaker but significant reactivity was observed with the peptide corresponding to residues 12 to 35, while the six remaining peptides showed no reactivity with HYB76-8.

For more precise localization of the reactive epitope, truncated peptides were synthesized. The reactivities of 15 peptides are shown in Fig. 2. The peptide designation is indicated according to the residues present.

Strong reactivity was observed with 10 of the peptides, with a striking decrease in ODs from peptide p3-15 to p3-14 (0.735 and 0.062, respectively). The corresponding ODs for p3-15, p4-15, and p5-15 were 0.735, 0.049, and 0.039 respectively. These findings thus indicate a requirement of the 13 EQQWNFAGIEAAA residues for full reactivity with MAb HYB76-8.

Peptide	Sequence	Reactivity with HYB76-8
p1-20	MTEQQWNFAGIEAAASAIQG	+++
p1-19	MTEQQWNFAGIEAAASAIQ	+++
p1-18	MTEQQWNFAGIEAAASAI	+++
p2-20	TEQQWNFAGIEAAASAIQG	+++
p3-20	EQQWNFAGIEAAASAIQG	+++
p3-19	EQQWNFAGIEAAASAIQ	+++
p3-18	EQQWNFAGIEAAASAI	+++
p3-17	EQQWNFAGIEAAASA	+++
p3-16	EQQWNFAGIEAAAS	+++
p3-15	EQQWNFAGIEAAA	+++
p3-14	EQQWNFAGIEAA	-
p3-13	EQQWNFAGIEA	-
p3-12	EQQWNFAGIE	-
p4-15	QQWNFAGIEAAA	-
p5-15	QWNFAGIEAAA	-

FIG. 2. Mapping of the HYB76-8-reactive epitope in ELISA through reaction with synthetic peptides. The core peptide is boxed.

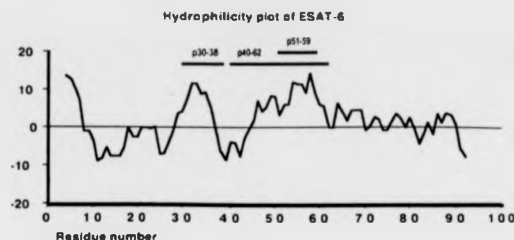


FIG. 3. Hydrophilicity plot of ESAT-6. The hydrophilicity value at each point is the sum of the results for seven consecutive amino acids blotted at the middle residue. The horizontal bars indicate the positions of the synthetic peptides used for immunization.

This conclusion was supported by testing of eight additional peptides (p3-11, p3-10, p4-14, p5-20, p5-14, p6-14, p7-14, and p8-14), which gave negative reactions with ODs of <0.050 in plates in which a positive control with peptide p1-20 gave ODs of >0.850.

Reactivity of anti-peptide antibodies with ESAT-6. Having established the location of the HYB76-8-reactive epitope, we continued our work to develop antibodies to other parts of the molecule. Figure 3 shows a hydrophilicity plot of ESAT-6. In addition to the N-terminal region, there are two other main hydrophilic areas which would be expected to contain surface-exposed areas of the polypeptide chain with B-cell epitopes. Consequently, two peptides, corresponding to positions 30 to 38 and 51 to 59, were synthesized with a cysteine residue added at the N-terminal end for coupling to KLH as the carrier molecule for immunization. In three rabbits immunized with one of the peptides coupled to KLH, antibodies were rapidly formed and reacted with the corresponding free peptide bound on a solid phase in an ELISA; however, no reactivity was observed with the other peptide. Two weeks after the second immunization, the titer in an ELISA was >1:1,000 with ODs at a 1:100 dilution of >0.850 with the relevant peptide in five rabbits and <0.035 with the other peptide.

The anti-p30-38 antibody reacted significantly with three of the overlapping peptides of ESAT-6; the strongest reaction was with peptide P3 containing residues 22 to 45. The anti-p51-59 antibody reacted with the P4 and P5 peptides; the strongest reaction was observed with P4 containing residues 32 to 55, as shown in Table 1. However, these antibodies did not

TABLE 1. Reactivity of antibodies to synthetic peptides of ESAT-6 in ELISA

Peptide	OD values		
	Anti-p30-38*	Anti-p51-59	Anti-p40-62
P1 (1-20)	0.049*	0.040	0.059
P2 (12-35)	0.136	0.050	0.066
P3 (22-45)	0.499	0.040	0.043
P4 (32-55)	0.141	0.877	0.350
P5 (42-65)	0.049	0.688	1.147
P6 (52-75)	0.046	0.066	0.681
P7 (62-85)	0.029	0.030	0.049
P8 (72-95)	0.033	0.030	0.047
rESAT-6	0.147	0.106	0.394

* Each antiserum shown is representative for three rabbits immunized with each peptide.

* The OD value recorded is for the second bleeding of each rabbit.

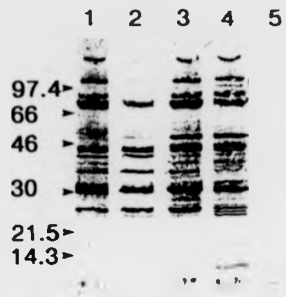


FIG. 4. Reactivity of the anti-p40-62 antipeptide antibody with *M. tuberculosis* culture fluid in Western blotting. Lanes: 1, serum obtained before immunization; 2 to 4, serum obtained 3, 11, and 15 weeks, respectively, after immunization; 5, reaction of MAb HYB76-8 with ESAT-6 for comparison. The positions of molecular mass markers are indicated at the left. The strong band at 30 kDa represents the antigen 85 complex.

react with native ESAT-6 in Western blotting or in capture ELISA with HYB76-8 at the solid phase, *M. tuberculosis* culture fluid or rESAT-6 in the second layer, the antipeptide antibody in the third layer, and the standard detection system for binding of rabbit immunoglobulin.

We therefore continued by immunizing three rabbits with a longer peptide corresponding to the extended hydrophilic region furthest from the HYB76-8 reactive epitope. A 23-mer peptide containing residues 40 to 62, containing the main hydrophilic region with neutral and hydrophobic residues on both sides, was selected. In ELISA with the peptide at the solid phase, significant antibody activity was demonstrated in serum taken 1 week after the second immunization. Reactivity in the ELISA with the overlapping peptides of ESAT-6 is shown for a representative antiserum (K618) in Table 1, with maximal activity as would be expected against peptide P5. Sera from early bleedings were again without reactivity with native ESAT-6 in Western blotting experiments or the capture ELISA. The immunization was therefore continued, and in late bleedings the antibodies also reacted with native ESAT-6 in Western blotting experiments, as shown in Fig. 4. In ELISA with rESAT-6 from *E. coli* at the solid phase, reactivity was low initially and markedly higher from the third bleeding onward; this is similar to the findings in Western blotting experiments. Figure 4 also illustrates the reactivity of rabbit serum obtained prior to immunization with several components of *M. tuberculosis* culture fluid, including the antigen 85 complex.

Double-antibody ELISA for quantification of ESAT-6. Having obtained antibodies reacting with two different epitopes on ESAT-6, titer determinations were performed to obtain the optimal conditions for an ELISA. For MAb HYB76-8, we selected a dilution (1:100) ensuring its presence in antibody excess in a reaction with *M. tuberculosis* culture fluids. Antigen preparations in the second layer were tested in twofold serial dilutions. Culture fluids and sonicates started at 4 μ g of total protein/well, isolated proteins tested for cross-reactivity in this system started at 4 μ g of protein/well, and purified rESAT-6 started at 0.1 μ g/well. The anti-p40-62 antiserum was used as a 1:200 dilution to ensure a sufficient strength of this antibody while avoiding the effects of coexisting antibodies to proteins of the antigen 85 complex in normal rabbit sera (Fig. 4), which tend to give rise to unwanted signals when more concentrated serum samples are used. Typical results are shown in Fig. 5.

M. tuberculosis H37Rv culture fluid gave a curve with a plateau at the three initial dilutions with ODs of about 1.20 and then decreasing. The curve of rESAT-6 isolated from *E. coli* initially decreased slowly; this was followed by a portion of the curve with an angle similar to that of the *M. tuberculosis* culture fluid curve. BCG Tokyo culture fluid gave no OD signals above 0.100 at any dilution, in agreement with the previous demonstration of the lack of this antigen in BCG. MAb HYB76-8 has previously (16) been demonstrated to cross-react with MPT64 and the antigen 85 complex. Purified MPT64 gave an OD of 0.093 at 4 μ g/well, and MPT59 (85B) gave a value of 0.084, both with entirely flat curves upon further dilution in the ELISA. The cross-reactivity of HYB76-8 thus had no significant influence on the assay system developed.

Application of double-antibody ELISA to recombinant microorganisms. Three rVV-ESAT-6 constructs were made with pSC11 initially and then p1108. p1108 was used to create rVV both with and without tPA. The two rVV constructs without tPA were ostensibly identical and included the same protein-coding sequence and promoter. The latter construct was included as a control for the p1108-derived rVV-tPA-ESAT-6. Successful incorporation of *esat-6* into VV was confirmed by plasmid sequencing and PCR (data not shown) or Southern blotting (Fig. 6A). This figure shows signals indicating *esat-6* in both digested pSC11-ESAT-6 and rVV-ESAT-6 and no signal for empty vector constructs. ESAT-6 transcription was also readily identified by RT-PCR (Fig. 6B). In this experiment, relevant controls excluded the possibility of contamination from the viral genome acting as the template. Despite confirming sequence fidelity and the presence of mRNA, protein expression in immunoblotting could not be identified by Western blotting (Fig. 6C). This absence of signal was true for the three separate rVV constructs with MAb HYB76-8 at a wide range of dilutions, blotting in the presence or absence of various blocking reagents, and the use of "preparatory" large-scale SDS-PAGE. ST-CF from *M. tuberculosis* was used as a positive control, and ESAT-6 was readily identified (Fig. 6C) in ST-CF diluted 1:100 containing approximately 10 to 100 ng of ESAT-6. Of note, nonspecific background was particularly apparent in lane 1, which has a high protein content. However, the background was limited to the higher-molecular-mass proteins and would not have interfered with a signal of 6 kDa.

Total-cell lysates (containing viral and cellular proteins) from rVV-infected cells were assessed by the capture ELISA:

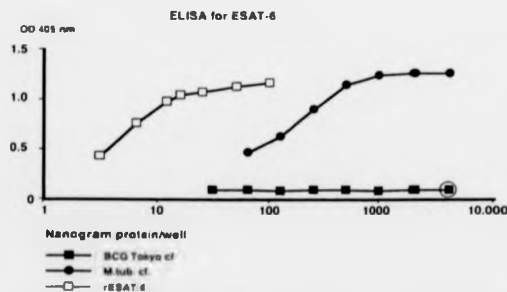


FIG. 5. Reactivity of *M. tuberculosis* culture fluid (M.tub. cf.) and purified recombinant ESAT-6 in the double-antibody ELISA, compared with BCG Tokyo culture fluid as negative control. The open circle indicates that corresponding ODs were obtained with the isolated proteins MPT64 and MPT59 (MPT59).

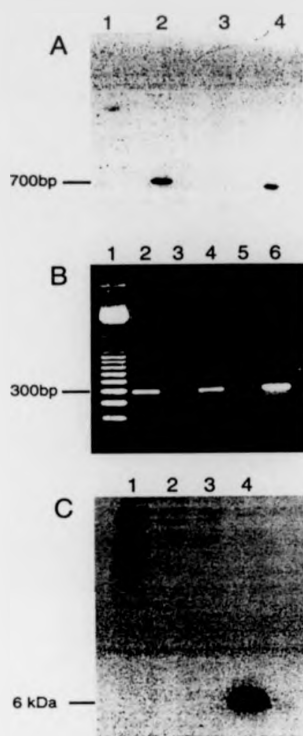


FIG. 6. (A) Southern blot of *EcoRI*-digested plasmid and purified viral DNA. Lanes: 1, nonrecombinant insertion plasmid pSC11; 2, pSC11-ESAT-6; 3, rVV- β -galactosidase; 4, rVV-ESAT-6. (B) RT-PCR of virally encoded ESAT-6 transcripts. Lanes: 1, 100-bp pair molecular mass marker; 2, DNase-treated, RT step included; 3, DNase treated, no RT step; 4, no DNase, no RT step (contaminating viral DNA); 5, no DNase, no RT step; 6, plasmid containing *esat-6* gene (positive control). (C) Western blot of rVV-infected cell lysates in a large-gel system with 285 μ l of cell lysate/well. Lanes: 1, neat cell lysate; 2, diluted 1:2; 3, 1:4; 4, *M. tuberculosis* culture filtrate (positive control).

twofold serially diluted samples were tested as illustrated in Fig. 7. The three rVV constructs illustrated were from monolayers with similar confluence and rVV titers, and the highest concentration corresponded to lysates of 4×10^3 cells. The lysate of cells containing rVV-IPA-ESAT-6 gave a strong signal. Upon dilution, the curve showed an angle similar to that for *M. tuberculosis* culture fluid, requiring about a 20-fold-higher total-protein content to give an ESAT-6 signal of similar strength. Lack of tPA in the rVV-ESAT-6 construct did not affect viral replication, in that PFU counts postinfection were similar for the two constructs. The construct containing *esat-6* alone, rVV-ESAT-6, gave a considerably weaker signal, still considered positive, while the lysate of cells with empty vector gave a flat curve.

DISCUSSION

In the experiments involving ELISA with overlapping peptides of the ESAT-6 sequence at the solid phase, the HYB76-8

reactive epitope was localized to the N-terminal part of the polypeptide chain (Fig. 1), with further localization to a core area with the EQQWNFAGIEAAA residues at positions 3 to 15 by the use of synthetic truncated peptides from this region, as shown in Fig. 2.

Comparing the reactivity illustrated in these figures, a striking feature emerges: The C-terminal border of the epitope is revealed by the striking difference in HYB76-8 reactivity with peptides p3-15 and p3-14, giving ODs in the ELISA of 0.735 and 0.062, respectively. In assays with the overlapping peptides, the ODs for peptides P1, P2, and P3 were 0.918, 0.186, and 0.009, respectively, thus showing a positive reaction with peptide P2 containing only four residues of the core epitope, EAAA. This indicates a striking influence of further flanking residues in the P2 peptide for maintenance of reactivity with MAb HYB76-8.

The mapping of the HYB76-8-reactive epitope to residues 3 to 15 corresponds exactly to the previous mapping of T-cell-reactive epitopes on this molecule in mice by Brandt et al. (7). Two T-cell epitopes, recognized in the context of different *H-2* types, were defined. In BALB/c (*H-2^d*) and C57BL/6j (*H-2^b*) mice, only the N-terminal peptide 1 of nine overlapping peptides covering the ESAT-6 sequence reacted with T cells recovered during recall of immunity to *M. tuberculosis*. Mapping with truncated synthetic peptides in C57BL/6j mice showed a slight variation in different test systems, defining the epitope to a 13-amino-acid core sequence corresponding to residues 3 to 15 in tests of IFN- γ release upon stimulation of T cells from memory immune mice.

This localization of reactivity was observed in only two of the six inbred strains tested, and we note that MAb HYB76-8 was generated in CF1 \times BALB/c F₁ mice (20) expressing *H-2^d*. Following infection with *M. tuberculosis* (8) or *M. bovis* BCG (18), formation of antibody to components of mycobacterial sonicates and culture fluids varies markedly, depending on the genes in the major histocompatibility complex. Additional work is needed to see whether B-cell epitope localization on a single protein, like ESAT-6, will differ in different inbred mouse strains.

While B-cell epitopes in general correspond to surface-exposed areas of the polypeptide chain and T-cell epitopes depend on antigen processing and presentation by the major histocompatibility complex, it is well established that areas of polypeptide chains may overlap with regard to the presence of B- and T-cell epitopes. Regarding mycobacterial proteins, this feature has been demonstrated in several instances. In the *M. tuberculosis* 16-kDa antigen, two linear B-cell epitopes at

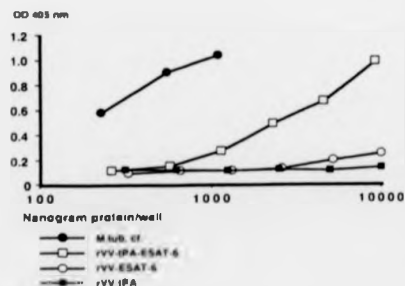


FIG. 7. Reactivity of double-antibody ELISA for ESAT-6 with rVV. M tub. cf., *M. tuberculosis* culture fluid.

p31-40 and p61-70 were demonstrated in mice (33), while three T-cell epitopes, one of them at p21-40, were demonstrated in human T-cell responses (11). A significant overlap of B- and T-cell epitopes has been demonstrated in the 19-kDa lipoprotein (6). Overlapping B-cell and T-cell epitopes were demonstrated regarding reactivity with the 65-kDa heat shock protein in cells obtained from patients with Behcet's disease (10). Pollock et al. (29) have demonstrated two T-cell epitopes, at positions 118 to 135 and 174 to 194 with numbering starting at Met 1 of the signal peptide, on the secreted MPB70 protein using cells from cattle infected with *M. bovis*. One of these, at the C-terminal end of the polypeptide chain, overlaps the B-cell epitope at position 174 to 190, reacting with the monoclonal antibody 1-1D (38). Billman-Jacobe et al. (6a) demonstrated another T-cell epitope on MPB70, at positions 103 to 113, overlapping the MAb 1-5C reactive epitope at positions 109 to 118 (38).

Reaction of our initial antipeptide antibodies with the peptide used for immunization without reactivity with the native ESAT-6 molecule is a well-known feature of antipeptide antibodies. When a longer peptide corresponding to the extended hydrophilic region of ESAT-6 was used, the fine specificity of the antipeptide antibodies changed upon extended immunization, and reactivity with native ESAT-6 was obtained, permitting the establishment of a double-antibody ELISA and giving a strong signal with *M. tuberculosis* H37Rv culture fluid. BCG Tokyo culture fluid was considered a particularly suitable negative control, containing proteins of the antigen 85 complex as well as large amounts of MPB64 (15), which was previously demonstrated to cross-react with HYB76-8 (16). Its signal was sufficiently low, giving a flat curve as illustrated in Fig. 5, thus demonstrating a marked difference in reactivity with respect to *M. tuberculosis* and BCG.

Observing the strong signal obtained in the double-antibody ELISA with lysates of cells containing rVV-tPA-ESAT-6 (Fig. 7), it was surprising to obtain no signal in Western blotting with MAb HYB76-8 on the same lysates, as shown in Fig. 6C. This observation is probably explained by the tendency of MAbs to depend strongly on the three-dimensional structure of the antigen and its reactive epitope while polyclonal antibodies are less sensitive in this regard.

A variety of *M. tuberculosis* preparations are being tested. ST-CF (3) gives a good signal, albeit somewhat weaker than that of culture fluids obtained from longer-lasting stationary-phase cultures of *M. tuberculosis* like the 4-week culture shown in Fig. 5. Culture fluids from 10 clinical isolates of *M. tuberculosis* gave strong signals, with all but one being stronger than those of the ST-CF preparations. In *M. tuberculosis* sonicates, a positive signal was obtained although it varied in strength. This corresponds to the demonstration of ESAT-6 in culture fluid as well as cytosol of *M. tuberculosis* by Sørensen et al. (30). A thorough investigation of culture fluids and sonicates of washed *M. tuberculosis* cells obtained from individual cultures is needed for further characterization of the release of the ESAT-6 protein from the mycobacterial cell (35).

Furthermore, the assay will be valuable for the demonstration and quantification of ESAT-6 produced by various recombinant organisms like BCG, *Mycobacterium smegmatis*, and VV to select candidate vaccines for protection studies. Information on ESAT-6 expression in different constructs of the same carrier is readily obtained, as illustrated in Fig. 7. Quantitative information comparing the expression in different carriers is more uncertain, since the sensitivity of the assay may vary for recombinant ESAT-6 in different carriers and in relation to native ESAT-6 in *M. tuberculosis*.

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Human cytolytic and interferon γ -secreting CD8⁺ T lymphocytes specific for *Mycobacterium tuberculosis*

AJIT LALVANI*†, ROGER BROOKES*†‡, ROBERT J. WILKINSON§¶, ADAM S. MALIN||, ANSAR A. PATHAN*, PETER ANDERSEN**, HAZEL DOCKRELL||, GEOFFREY PASVOL¶, AND ADRIAN V. S. HILL*

*Molecular Immunology Group, Institute of Molecular Medicine, Nuffield Department of Clinical Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom; †Tuberculosis and Related Infections Unit, Royal Postgraduate Medical School, London W12 0NN, United Kingdom; ‡Department of Infection and Tropical Medicine, Imperial College School of Medicine, Northwick Park Hospital, London HA1 3UJ, United Kingdom; §Clinical Sciences, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E, United Kingdom; and ¶Tuberculosis Unit, Statens Serum Institut, Artillerivej 5, Copenhagen-S, Denmark DK2300

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ABSTRACT Protective immunity to *Mycobacterium tuberculosis* is poorly understood, but mounting evidence, at least in animal models, implicates major histocompatibility complex class I-restricted CD8⁺ T cells as an essential component. By using a highly sensitive assay for single cell interferon γ release, we screened an array of *M. tuberculosis* antigen-derived peptides congruent with HLA class I allele-specific motifs. We identified CD8⁺ T cells specific for epitopes in the early secretory antigenic target 6 during active tuberculosis, after clinical recovery and in healthy contacts. Unrestimulated cells exhibited peptide-specific interferon γ secretion, whereas lines or clones recognized endogenously processed antigen and showed cytolytic activity. These results provide direct evidence for the involvement of CD8⁺ cytotoxic T lymphocytes in host defense against *M. tuberculosis* in humans and support current attempts to generate protective cytotoxic T lymphocyte responses against *M. tuberculosis* by vaccination.

One-third of the world's population is latently infected with *Mycobacterium tuberculosis*, which causes over 2 million deaths per year (1); more than any other single pathogen. The global resurgence of tuberculosis, together with the HIV pandemic and emerging multi-drug resistance, have heightened the need for an effective vaccine suitable for use in immunocompromised individuals. The current live attenuated vaccine, Bacille Calmette-Guerin (BCG), has variable and limited efficacy in tuberculosis-endemic regions (2). Knowledge of protective immune responses in tuberculosis is incomplete. Delayed type hypersensitivity, mediated by cytokine-secreting CD4⁺ T cells, contributes to both pathogenesis and protection. However, delayed type hypersensitivity, as measured by tuberculin skin testing, does not correlate with the partial protection induced by BCG vaccination, implicating another component of cell-mediated immunity (2). CD8⁺, as well as CD4⁺ T cells are a feature of the tuberculous granuloma. Recent evidence from murine models points to a protective role for CD8⁺ T cells in *M. tuberculosis* infection (3–6). However, major histocompatibility complex (MHC) class I-restricted CD8⁺ T cells specific for *M. tuberculosis* have not hitherto been found in humans, although BCG-specific CD8⁺ T cell responses of undefined antigen specificity have been reported (7). Low frequencies of MHC class I-restricted *M. tuberculosis*-specific CD8⁺ cytotoxic T lymphocytes (CTL) below the limits of detection of standard assays may have impeded their detection; we have adopted a novel, sensitive approach to identify such cells.

Two secreted antigens of *M. tuberculosis*, early secretory antigenic target 6 (ESAT-6) and the Antigen 85 complex, were studied because secreted antigens are implicated as targets of protective immune responses (8) and are more likely to access the host cell cytosol and hence the MHC class I antigen processing pathway. These antigens are the target of protective immune responses in mice (9, 10). ESAT-6 (6 kDa) is virtually specific for *M. tuberculosis* complex; the gene is present in all isolates of *M. tuberculosis* and virulent *Mycobacterium bovis* but is absent from all strains of BCG examined (11). The 30–32-kDa Antigen 85 complex consists of 3 homologous proteins, A, B, and C, which constitute 30% of *M. tuberculosis* culture filtrate (12) and elicit strong CD4⁺ T cell responses in tuberculin skin test-positive subjects (13).

The sequences of ESAT-6 and antigens 85A, 85B, and 85C were scanned with allele-specific peptide motifs for the HLA class I types HLA-A2, HLA-B7, HLA-B8, HLA-B35, HLA-B52, and HLA-B53, all of which were present in the study population. A total of 49 peptides congruent with these motifs were synthesized (Table 1). These candidate epitopes were then sorted into pools according to HLA class I alleles and used to stimulate peripheral blood mononuclear cells (PBMCs) from subjects with the corresponding HLA class I allele. *In vitro*-expanded populations of peptide-specific T cells were then detected with two different assays of effector function. In a preliminary study using the standard ⁵¹Cr release CTL assay we detected low levels of lytic activity against some peptide pools, but responses from the few remaining cells were too weak to define epitopes. We therefore adapted a more sensitive enzyme-linked immunospot (ELISPOT) technique for detecting single cell interferon γ (IFN- γ) secretion. We calibrated this assay against the ⁵¹Cr release CTL assay and found it an order of magnitude more sensitive for detecting low numbers of cultured human CD8⁺ peptide-specific T cells (14).

Thirty-nine adults were studied using the more sensitive ELISPOT assay. These included patients representing the full clinical spectrum of active tuberculosis (pulmonary, lymphadenitis, and extrapulmonary), as well as healthy contacts.

MATERIALS AND METHODS

Subjects. Thirty-nine adult patients and contacts with suitable HLA haplotypes were studied. Most subjects originated

This paper was submitted directly (Track 11) to the *Proceedings* office. Abbreviations: BCG, Bacille Calmette-Guerin; MHC, major histocompatibility complex; CTL, cytotoxic T lymphocyte; ESAT-6, early secretory antigenic target 6; PBMCs, peripheral blood mononuclear cells; IFN- γ , interferon- γ ; SFC, spot-forming cells; STCL, short-term cell line; BCL, B cell line; ELISPOT, enzyme-linked immunospot. †A.L. and R.B. contributed equally to this work.

‡To whom reprint requests should be addressed. e-mail: roger.brookes@ndm.ox.ac.uk.

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Table 1. Peptides from ESAT-6

HLA class I allele	Peptide motif	Peptide	Sequence	Position
HLA-A2	-L/I/M-----V/L/I	ES8	GIEAAASA	10-18
		ES9	AIQGNVTSI	17-25
		ES10	LLDEGKQSL	28-36
		ES11	ELNNALONL	64-72
		ES13	AMASTEGNV	82-90
HLA-B8	-K-K---L/I	ES7	EGKSLTKL	31-39
HLA-B52	-Q-----I/V	ES12	LQNLARTI	69-76

We searched for 8-, 9-, or 10-residue sequences from ESAT-6, which were congruent with the peptide motifs of the HLA class I alleles -A2, -B7, -B8, -B35, -B52, and -B53. Sequences congruent with the peptide motifs for HLA-A2, HLA-B8, and HLA-B52 were identified; these peptides were synthesized and are displayed. No sequences congruent with HLA-B7, HLA-B35, and HLA-B53 were present in ESAT-6 and, thus, no peptides were synthesized for these HLA class I alleles. Peptides were sorted into pools that were used for *in vitro* restimulation of donor PBMCs. Peptides found to be CD8⁺ epitopes are shown in boldface. Similarly, 42 peptides were synthesized based on the sequences of antigens 85A and 85C. No CD8⁺ epitopes were identified among these and the peptides are not shown.

from the Indian subcontinent, Africa, or northern Europe. The number of patients with different clinical types of disease, as well as the number of healthy contacts, are listed in Table 2. Most subjects were tuberculin skin test positive. All contacts, except one who was not tested, had a positive Heaf test of grade 2 or more. All patients had at least 5 mm of cutaneous induration in response to intradermal injection with 1 tuberculin unit of purified protein derivative, except four who were not tested and four who were negative (three with old, inactive pulmonary tuberculosis and one with active miliary disease). The distribution of each of the 6 HLA class I alleles for which we tested peptide pools was as follows: HLA-A2 was present in 26 subjects, HLA-B8 in 7 subjects, HLA-B7 in 6 subjects, HLA-B35 in 2 subjects, HLA-B52 in 2 subjects, and HLA-B53 in 2 subjects.

Each subject was tested against the peptide pool (or pools if the subject had more than one suitable HLA class I allele) once only. Repeat blood samples were obtained and further testing carried out only if the donor had responded on the first occasion.

Diagnosis of Tuberculosis in Patients NPH54 and NPH97. NPH54 was diagnosed with tuberculosis on the basis of clinical symptoms, acute erythema nodosum and mediastinal lymphadenopathy. Tuberculin skin test showed 25 mm induration 48 hr after intradermal injection of 1 tuberculin unit of purified protein derivative. In addition, a very high antibody titer of > 1/6,250 in response to the 16 kDa antigen of *M. tuberculosis*, helped to confirm active infection (15). For NPH97, the tuberculin skin test was positive and culture of a proximal phalangeal bone biopsy of the affected hand on Lowenstein-Jensen medium grew *M. tuberculosis*.

Tissue Typing. Subjects were HLA typed serologically. NPH54, NPH97, WB, Akiba, and PG were additionally typed

Table 2. Numbers of healthy contacts and patients with different types of clinical disease

Clinical phenotype	Subjects, n
Pulmonary (active)	8
Pulmonary (inactive)	6
Pleural	2
Lymphadenitis	4
Osteoarticular	5
Miliary	1
Gastrointestinal	1
Epididymo-orchitis	1
Healthy contacts	11

by amplification refractory mutation system (ARMS)-PCR using sequence-specific oligonucleotide primers (16).

Peptide Synthesis. Peptides were synthesized on solid phase on a synthesizer (Zinsser, Frankfurt) using fluorenylmethoxycarbonyl chemistry. Purity was confirmed by HPLC.

ELISPOT Assay for IFN- γ . Ninety-six-well polyvinylidene difluoride (PVDF)-backed plates (Millipore) precoated with the anti-IFN- γ mAb 1-D1K at 15 μ g/ml (MABTECH, Stockholm) were washed with RPMI medium 1640 and blocked with R10 for 1 hr at room temperature. Short-term cell lines (STCLs), CTL lines, or clones were washed two times in RPMI medium 1640, resuspended in R10, and dispensed at known input cell numbers per well in duplicate wells. Peptide was added either directly to the supernatant at a final concentration of 2 μ M (free peptide) or presented on a B cell line (BCL), prepulsed with 10 μ M peptide for 1 hr at 37°C, and then washed three times in R10. Plates were incubated for 12 hr at 37°C, in 5% CO₂/95% air. After washing six times with phosphate buffered saline (PBS)/0.05% Tween 20 to remove cells, plates were incubated for 3 hr with the second biotinylated anti-IFN- γ mAb 7-B6-1-biotin at 1 μ g/ml (MABTECH). A further wash, as above, was followed by incubation with a 1:1,000 dilution of streptavidin-alkaline phosphatase conjugate for 2 hr. After another wash, chromogenic alkaline phosphatase substrate (Bio-Rad) was added to the wells, and 30 min later plates were washed with tap water. After drying, spot-forming cells (SFC) were counted under $\times 20$ magnification. For *ex vivo* ELISPOT assays, 500,000 freshly isolated uncultured PBMCs were used per well. Responses were considered significant if a minimum of 10 SFCs were present per well and, additionally, this number was at least twice that in control wells.

Generation of CD8⁺ T Cell Lines. STCLs were generated as described (17). Briefly, PBMCs separated from whole blood were prepulsed with 30–50 μ M peptide for 1 hr in a cell pellet and then diluted up to 1×10^6 cells/ml in RPMI medium 1640 supplemented with 10% pooled heat-inactivated human AB serum (R10) and 25 ng/ml rhIL-7 (R & D Systems), and seeded at 200 μ l per well in a U-bottomed 96-well plate. Lymphocult-T (Biotest, Dreieich, Germany) was added to 10% of each well at regular intervals. STCLs were assayed at day 14. Lines 3–9, 3–10, and 3–20 were generated from individual STCLs by two rounds of restimulation (on days 14 and 21) with autologous peptide-pulsed irradiated BCL at a 1:3 feeder:responder ratio. Line 4–1 was generated by pooling four ES12-specific STCLs (originally established with CD4-depleted PBMCs), followed by restimulation with peptide-pulsed autologous BCL.

Generation of CD8⁺ T Cell Clones. Enumeration of IFN- γ SFCs after CD4 and CD8 depletions indicated that the frequency of CD8⁺ ES12-specific T cells in NPH54-derived monospecific lines 3–9 and 3–20 was 1:44 and 1:67 CD8⁺ T cells, respectively. This indicated that for T cells seeded at one cell per well, 1.5–2.3 specific clones should be generated per 100 wells seeded. This quantitative data guided our cloning procedure: for each line, 240 wells were seeded at one cell per well (following CD4 depletion). Cloning mix consisted of three-way mixed lymphocyte reaction, 10% Lymphocult-T, phytohemagglutinin at 1 μ g/ml, and ES12-pulsed autologous irradiated washed BCL, with a total of 100,000 feeders per well. Clones were screened in ELISPOT assays and three ES12-specific clones were subsequently recovered from line 3–9 and two from line 3–20. Clones were maintained by peptide-pulsed autologous irradiated BCL restimulation and supplementation with 10% Lymphocult-T and rhIL-7 at 25 ng/ml.

⁵¹Cr Release Cytotoxicity Assays. Standard chromium release assays were performed as described (18). In brief, BCLs were labeled with 100 mCi ⁵¹Cr (Amersham), washed in RPMI medium 1640, and then pulsed with peptide as above and

plated out at 5,000 cells per well. CTL, R10, or 5% Triton X-100 were added. Test wells were in duplicate, other wells in quadruplicate. Plates were incubated for 5 hr at 37°C, in 5% CO₂/95% air and harvested supernatant read on filtermats in an LKB 1205 beta-plate scintillation counter (Wallac, Gaithersburg, MD). Background ⁵¹Cr release was less than 20%. Percent lysis was calculated from the formula $100 \times (E - M)/(T - M)$, where E is the experimental release, M is the spontaneous release, and T is the maximal release.

Specific Cell Depletions. CD4⁺ and CD8⁺ T cells were depleted by 30-min incubation with anti-CD4 or anti-CD8 mAbs conjugated to ferrous beads, DYNABEADS M-450, (DynaL, Oslo) in 500 µl of R10 on ice. Following dilution of up to 5 ml in R10, the conjugate-coated cells were removed by a magnetic field. CD8⁺ T cell depletions were highly effective and were not toxic, because there was no detectable loss of viability in the depleted population and responses of antigen 85-specific CD4⁺ T cell lines were unaffected by depletion.

Recombinant Vaccinia Virus Construction. Construction of the rVV is described in detail elsewhere (ref. 19; and A.S.M., unpublished work). In brief, the amplification product of the ESAT-6 gene obtained by PCR using the plasmid template pAA249 (P. Andersen, Statens Serum Institut, Copenhagen-S, Denmark) was cloned into plasmid p1108-tPA to create p1108-tPA-ESAT-6. Homologous recombination into the thymidine kinase locus of vaccinia strain WR with cationic lipid transfection was followed by selection of rVV using mycophenolic acid. A second negative control rVV was also constructed by using p1108 minus the coding sequence. Recombinants were verified by sequencing and expression confirmed by PCR and capture ELISA (M. Harboe, unpublished work).

RESULTS

Identification of ESAT-6-Specific Effector T Cells Direct from Peripheral Blood. Two CD8⁺ epitopes in ESAT-6 were identified. The T cells from donor NPH54, who had tuberculous mediastinal lymphadenitis, recognized peptides corresponding to both of these epitopes. Uncultured PBMCs isolated at the time of the diagnosis from NPH54, who has HLA-B52 and HLA-A2.01, secreted IFN-γ in response to an ESAT-6-derived peptide pool for these class I alleles in an *ex vivo* ELISPOT assay. The mean number of IFN-γ SFCs enumerated from 5×10^5 PBMCs in duplicate wells was 19 for the ESAT-6 peptides compared with 2 in the control wells with no peptide. A subsequent assay tested freshly isolated PBMCs against each of the individual peptides within the responding pools; IFN-γ SFCs were detected in response to peptides ES12 and ES13, whose sequences are congruent with the HLA-B52 and HLA-A2.01 peptide motifs, respectively. The frequency of ES12- and ES13-specific IFN-γ SFCs is of the same order of magnitude as SFCs for HLA-A2.01-restricted influenza matrix epitope M1 58–66 (Fig. 1A). Unrestimulated PBMCs from a second donor, NPH97, with tuberculous osteomyelitis, also recognized the ES12 peptide. This patient also has HLA-B52 and HLA-A2.01, and the magnitude of the ES12-specific response was similar to the response to the HLA-A2-restricted influenza matrix epitope. Single cell IFN-γ release by freshly isolated T cells in these short 12-hr *ex vivo* assays, employing no stimulus other than cognate peptide, indicates that these cells are highly likely to be circulating activated effector T cells (14).

ESAT-6 Epitope-Specific T Cells Are CD8⁺. ES12-specific T cell lines were generated from NPH54 and NPH97 PBMCs. Depletion experiments demonstrated that the ES12-specific T cells are CD8⁺ (Fig. 1B). Similar depletion studies on ES13-specific STCLs from donor WB, a healthy contact with HLA-A2.01, confirmed that these ES13-specific T cells are also CD8⁺ (Fig. 1C). CD4-depleted ES13-specific STCLs from donor NPH43, a patient with lymphadenitis (class I HLA

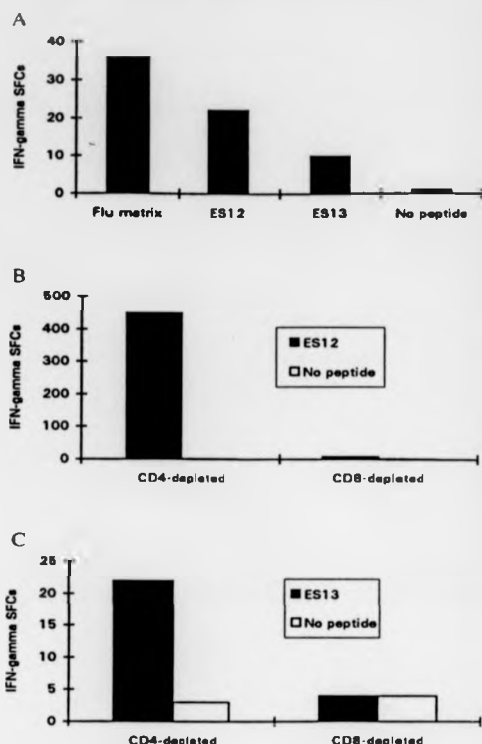


FIG. 1. (A) Enumeration of circulating peptide-specific effectors from the peripheral blood of NPH54 (HLA class I haplotype: HLA-A2.01, HLA-A68; HLA-B52, HLA-B38). The sequences of peptides ES12 and ES13 are congruent with the peptide motifs of HLA-B52 and HLA-A2.01, respectively. The frequency of ES12-specific effectors during active tuberculosis (1:23,000) is slightly less than the frequency of effectors specific for the HLA-A2-restricted epitope in influenza virus M1 58–66 (1:14,000). Activated effectors specific for ES13, an HLA-A2-restricted epitope, are also evident, but at a lower frequency (1:50,000). Freshly isolated PBMCs (500,000) were seeded in duplicate wells and peptide added to a final concentration of 2 µM in a 12-hr *ex vivo* ELISPOT assay for IFN-γ. (B) ES12-specific IFN-γ secreting T cells are CD8⁺. Enumeration of IFN-γ SFCs in a 12-hr ELISPOT assay for IFN-γ with cell line 3–20 from donor NPH54 was performed and is shown. After depletion of CD4⁺ or CD8⁺ cells, 20,000 cells were added to each of a pair of duplicate wells and peptide ES12 was added to a final concentration of 2 µM; the mean number of SFCs is shown. No SFCs were observed in the absence of peptide. CD8⁺ cell depletion completely abrogates the response. Similar results were obtained with cell line 3–9. (C) ES13-specific IFN-γ release by an STCL raised against ES13 with PBMCs from donor WB; a healthy contact with HLA haplotype HLA-A2, HLA-A1; HLA-B7, HLA-B8. After magnetic depletion of CD4⁺ or CD8⁺ cells, 20,000 cells were added to each of a pair of duplicate wells in a 12-hr ELISPOT assay and peptide added at 2 µM to the supernatant. The mean number of IFN-γ SFCs for each pair of wells is shown.

haplotype: HLA-A2.01, HLA-A29; HLA-B7, and HLA-B51), recognized peptide presented through HLA-A2.01 on ES13-prepulsed HLA-A2.01-matched heterologous BCL. Ninety-eight IFN-γ SFCs were enumerated in response to the ES13-pulsed BCL, compared with 48 IFN-γ SFCs for the unpulsed control BCL; the high backgrounds are probably due to

alloresponse (13). Responses to ES13 were transient and often undetectable in PBMCs from subsequent blood samples drawn later in the course of the therapy, thus precluding comprehensive analysis of this epitope.

No CD8⁺ epitopes were identified among any of the 42 peptides from antigens 85A, 85B, or 85C. Despite stimulation *in vitro* with nonamer peptides, the resultant STCLs were all CD4⁺. Interestingly, certain peptides elicited IFN- γ secretion by freshly isolated uncultured CD4⁺ cells in 12-hr *ex vivo* ELISPOT assays (A.L., R.B., R.J.W., G.P., and A.V.S.H., unpublished data.)

ES12-Specific CD8⁺ T Cells Are MHC Class I-Restricted and Recognize Endogenously Processed Antigen. The epitope ES12 was further characterized with ES12-specific lines and clones. T cell recognition of peptide had until now relied on presentation of ES12 on autologous cells by adding free peptide to the ELISPOT assay supernatant. To demonstrate HLA class I restriction, ES12 peptide was presented to clones with ES12-prepulsed BCL matched or mismatched at B52; only HLA-B52-matched ES12-prepulsed BCL were recognized by cells pooled from clones 3-1, 3-15, and 3-98 (Fig. 2A). ES12-specific lines raised from NPH97's PBMCs were similarly confirmed to be HLA-B52 restricted (data not shown).

To show that the ES12-specific CD8⁺ T cell clones were capable of recognizing endogenously processed antigen, autologous BCL infected with vaccinia virus recombinant for ESAT-6 (rVV-ESAT-6) or a control lacking the ESAT-6 sequence, were used to stimulate cytokine release. Only clones 3-1, 3-15, and 3-98 incubated with the ESAT-6 recombinant vaccinia-infected BCL secreted IFN- γ in the ELISPOT assay (Fig. 2B).

M. tuberculosis Antigen-Specific CD8⁺ T Cells Are Cytolytic. By using a sensitive measurement of cytokine release, we characterized CD8⁺ T cells specific for ES12. We now returned to conventional ⁵¹Cr release assays to test whether these cells were also capable of lytic activity. This was confirmed over a broad range of effector: target ratios for CD8⁺ T cell lines from both NPH54 and NPH97 using ES12-pulsed heterologous HLA-B52-matched BCL as targets (Fig. 3A and B). Peptide-specific lysis was detectable even when the peptide prepulse concentration was titrated as low as 1 nM (data not shown). Finally, to establish whether ES12-specific CTL could also kill targets expressing endogenously processed antigen, we demonstrated HLA-B52-restricted lysis of heterologous HLA-B52-matched targets infected with rVV-ESAT-6 (Fig. 3C).

DISCUSSION

We have identified CD8⁺ HLA class I-restricted T cells specific for epitopes in the *M. tuberculosis* protein ESAT-6 in 4 of 39 infected individuals. This is almost certainly an underestimate of the actual prevalence of *M. tuberculosis*-specific CD8⁺ CTL in infected individuals for the following reasons. First, we limited the search to epitopes restricted through six HLA class I alleles. Second, of the very large number of antigens secreted by *M. tuberculosis*, we studied only two. And third, *M. tuberculosis*-specific CD8⁺ CTL are more likely to be found in draining lymph nodes or at the site of infection rather than in the peripheral circulation. Nevertheless, the frequencies of circulating ESAT-6-specific effectors in the peripheral blood of NPH54 approximates to that for an influenza virus epitope (Fig. 1A). This relatively high frequency of CTL effectors specific for a single bacterial epitope is comparable to that found in malaria, a protozoal disease, where there is substantial indirect evidence for a protective role for *Plasmodium falciparum*-specific CTL (18, 20, 21). ESAT-6-specific effectors among uncultured PBMCs were detectable in the *ex vivo* ELISPOT assay in NPH54 with tuberculous lymphadenitis and in NPH97 with tuberculous osteomyelitis. For the common HLA class I allele, HLA-A2,

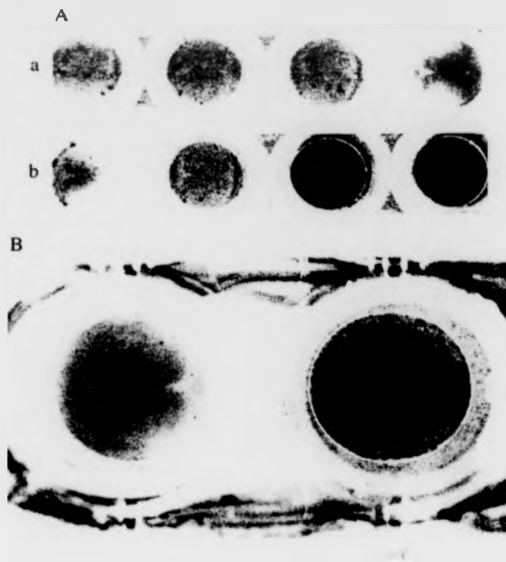


Fig. 2. (A) Photomicrograph showing peptide-specific HLA-B52-restricted IFN- γ release by ES12-specific CD8⁺ clones derived from donor NPH54. Cells from clones 3-1, 3-15, and 3-98 were pooled and assayed against unpulsed (first pair of wells in a and b), and ES12-pulsed (second pair of wells in a and b). HLA-B52-mismatched (A) and matched (B) target BCLs. The mismatched BCL is PG: HLA-A2.01, HLA-A3; HLA-B7, HLA-B51 and is shown in A. The matched BCL, a homozygous typing line, is Akiba: HLA-A24; HLA-B52, and is shown in B. Assays were performed in duplicate wells with 5,000 T cells and 50,000 B cells per well. Only the pair of duplicate wells with ES12-pulsed HLA-B52-matched targets are positive; the spots are so numerous that they appear confluent. (B) Photomicrograph showing IFN- γ release by ES12-specific CD8⁺ clone 3-15 of donor NPH54, in response to autologous BCL infected with vaccinia virus recombinant for the gene encoding ESAT-6 (rVV-ESAT-6). The negative control is the left-hand well, using autologous BCL infected with rVV control. BCL were infected the night before with the respective recombinant viruses at a multiplicity of infection (m.o.i.) of 7 plaque-forming units per cell in serum-free medium; after 90 min, cells were diluted up to 1 million/ml in R10 and incubated overnight. Infected BCL (100,000) were then added to each well along with 5,000 cloned T cells. The photomicrograph shows the result with clone 3-15, giving in excess of 450 SFCs. The results with the other two clones, 3-1 and 3-98, were so strongly positive that the spots were confluent. ($\times 20$.)

the number of subjects studied is sufficient to permit a preliminary comparison of the prevalence of responders between the different clinical subgroups. For donors with HLA-A2, ES13-specific IFN- γ secretion in freshly isolated PBMCs or STCLs was observed in 1 of 7 healthy contacts and 2 of 3 patients with lymphadenitis, but in none of 12 patients with other more disseminated forms of disease (pulmonary, pleural, and gastrointestinal). This distribution of responses suggests that responses to ESAT-6 may be associated with an immune response capable of containing *M. tuberculosis*, but larger numbers of subjects will be required to establish this.

The ELISPOT assay for IFN- γ release may measure an effector function of more protective relevance than the ⁵¹Cr release cytotoxicity assay. IFN- γ , a potent activator of macrophages, is essential for resistance to *M. tuberculosis* infection in mice (22, 23), whereas recent studies in perforin gene and Fas receptor gene knockout mice indicate that the lytic activity of CD8⁺ T cells is not required to control virulent *M.*

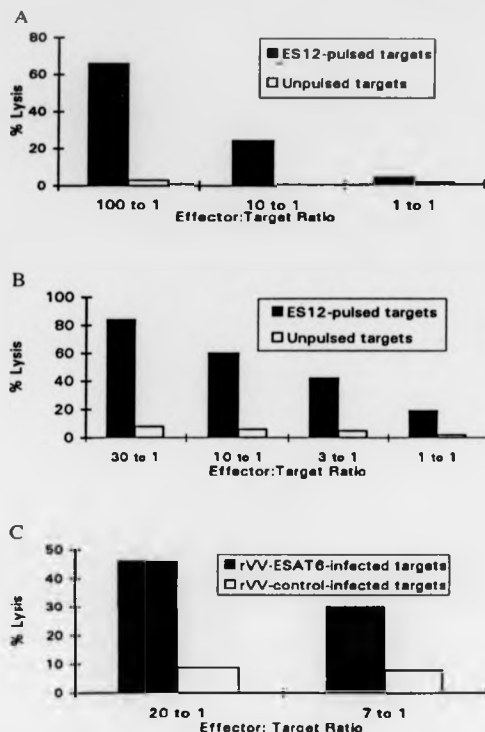


Fig. 3. (A) ES12-specific cytotoxic activity of CTL line 4-1 from donor NPH54 in a 5 hr ^{51}Cr release cytotoxicity assay. Peptide-specific lysis titrated downwards with diminishing effector-to-target cell ratio, and nonspecific lysis of unpulsed targets was less than 5%. Target cells were heterologous HLA-B52-matched BCL (homozygous typing line, Akiba; HLA-A24; HLA-B52, as in Fig. 2A), prepulsed with 10 μM ES12. (B) ES12-specific cytotoxic activity of a CTL line from donor NPH97 in a 5 hr ^{51}Cr release cytotoxicity assay. The CTL line was generated by restimulation of 14-day STCLs (cultured as described in *Materials and Methods*) with ES12-pulsed, washed, irradiated autologous BCL. Peptide-specific lysis titrated downwards with each 3-fold diminution in effector-to-target cell ratio, and nonspecific lysis of unpulsed targets was less than 5%. Target cells were heterologous HLA-B52-matched BCL (Akiba) as in Fig. 2A, prepulsed with 10 μM ES12. (C) Lysis of HLA-B52-matched heterologous targets (Akiba BCL) expressing endogenously processed ESAT-6. Targets were infected with rVV-ESAT-6 and rVV-control as in Fig. 2B and were labeled with ^{51}Cr the following day. CTL line 4-1 raised against ES12 specifically lysed the rVV-ESAT-6-infected targets; lysis of rVV-control-infected targets was below 10%.

tuberculosis infection (24). Moreover, humans homozygous for a point mutation in the IFN- γ receptor 1 gene, in whom cell surface expression of this receptor is absent, are highly susceptible to fatal disseminated mycobacterial infection (25), and human T cell-derived IFN- γ has recently been reported to inhibit the intracellular growth of *M. tuberculosis*.*

CD8 $^{+}$ CTL-derived IFN- γ may be especially important both for cells lacking MHC class II molecules, e.g., in the lung (26)

and for macrophages where mycobacteria can evade recognition during chronic infection by sequestering their antigen away from sensitized CD4 $^{+}$ T cells (27). Moreover, infection of murine macrophage cell lines with live *M. tuberculosis* has recently been shown to down-regulate MHC class II expression while simultaneously enhancing the presentation of exogenous soluble antigen through the MHC class I antigen processing pathway (28).

The observation that ES12-specific T cell lines and clones recognize target cells infected with vaccinia virus recombinant for ESAT-6 indicates that this antigen can be endogenously processed through the MHC class I antigen processing pathway, resulting in the presentation of the epitope ES12 through HLA-B52. Because responses to the *M. tuberculosis*-specific peptide ES12 were elicited from freshly isolated, unstimulated lymphocytes in an *ex vivo* assay, CD8 $^{+}$ T cells must have been primed through recognition of processed antigen *in vivo*. This study thus provides evidence that in humans an *M. tuberculosis* antigen is naturally processed *in vivo* through the MHC class I pathway leading to the induction of MHC class I-restricted effector T cells. Further support comes from preliminary data showing that human macrophages infected with *M. tuberculosis* *in vitro* are recognized by ES12-specific, HLA-B52-restricted CTL that suppress mycobacterial growth (R.B., et al., unpublished data).

Murine models, including studies with β_2 -microglobulin gene knockout mice (3), TAP-1 null mutant mice (28), and adoptive transfer experiments with HSP-65 immunized mice (4) show that CD8 $^{+}$ CTL are essential for protection against *M. tuberculosis* infection. CD8 $^{+}$ T cells also constitute a crucial effector mechanism in the protective immunity conferred by DNA vaccination against tuberculosis (9, 29). It has therefore been important to establish whether MHC class I-restricted CD8 $^{+}$ CTL play a role in *M. tuberculosis* infection in humans, for, if so, their induction could guide the rational design of subunit vaccines. However, it has proved very difficult to identify these cells in humans (7, 30). Indeed, the recent identification of human CD1-restricted CD8 $^{+}$ T cells specific for *M. tuberculosis* nonpeptide antigens has led to the suggestion that disruption of CD1-mediated antigen presentation may account for the enhanced susceptibility of β_2 -microglobulin gene knockout mice to *M. tuberculosis* infection (31). However, this study demonstrates the presence of classical MHC class I-restricted CD8 $^{+}$ CTL specific for an *M. tuberculosis* protein antigen in infected individuals. These cells circulate at a relatively high frequency in peripheral blood and freshly isolated, unstimulated cells rapidly display effector function within 12 hr of antigen contact. Induction of MHC class I-restricted peptide-specific CD8 $^{+}$ CTL with new generation CTL-inducing vaccines is now feasible. The phenotype and specificity of the cells identified here not only endorses efforts to develop CTL-inducing vaccines against tuberculosis but also supports the candidacy of ESAT-6 as a component of such vaccines.

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